Docket No.: SUPP-P01-011
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	Blau *et al.*	Confirmation No.:	1982
Application No.:	10/688,747	Art Unit:	1633
Filed:	October 16, 2003		
For:	METHODS FOR TREATING DISORDERS OF NEURONAL DEFICIENCY WITH BONE MARROW-DERIVED CELLS	Examiner:	Q. J. LI

DECLARATION UNDER 37 C.F.R. § 1.132 OF HELEN BLAU

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Helen Blau, of 580 Cotton Street, Menlo Park, CA 94025, hereby declare and state as follows:

1. I am a Professor in the Department of Microbiology and Immunology at Stanford University. I am also an inventor of the subject application. I have been conducting research in the field of molecular and stem cell biology for approximately thirty years. A copy of my *curriculum vitae* is enclosed herewith.

2. Figures 1a-2b show the results of experiments carried out by me or under my direction.

3. In Figure 1a and 1b, hematopoietic stem cells (HSC) or their progeny are shown to fuse to Purkinje neurons to form cerebellar heterokaryons. To isolate individual mouse HSC, bone marrow was isolated from a GFP+ mouse, and SPKLS HSCs were identified as side-population positive after Hoescht staining as well as antibody positive for c-kit, Sca-1, and antibody negative for the standard panel of lineage markers. A single GFP+ SPKLS HSC was injected into a lethally irradiated recipient along with GFP(-) Sca-1-depeleted bone marrow cells to aid in hematopoietic engraftment. In recipient mice in which the single SPKLS cell reconstituted the hematopoietic

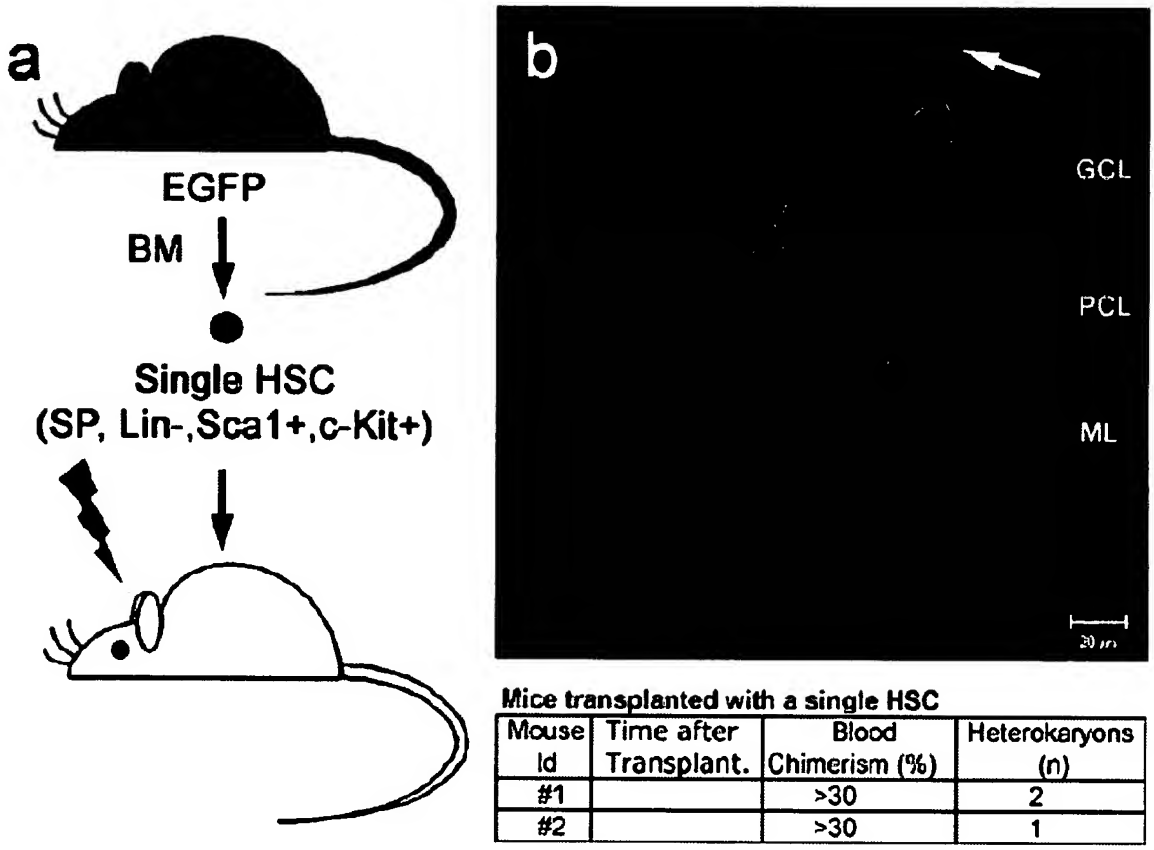
system, progeny of the single injected GFP+ SPKLS cells fused with adult cerebellar Purkinje neurons and formed stable binucleate cerebellar heterokaryons on the order of 1-2 per cerebellum (Fig. 1a & b). This experiment demonstrates the ability of an HSC to produce a fusion with Purkinje neurons to form cerebellar heterokaryons.

4. In Figures 2a and 2b, cell fusion is shown to be independent of variables associated with bone marrow transplantation. To demonstrate that formation of cerebellar heterokaryons was independent of the transplantation variables that may become associated with lethal total body irradiation, we used a well-established method, parabiosis, to introduce GFP+ blood into mice without exposure to irradiation (Fig. 2a). Parabionts are established by surgically joining two mice, the recipient wild-type mouse (no GFP expression) and a donor mouse expressing GFP as a transgene. Parabiotic animals develop a common anastomosed circulatory system within 3-10 days and reach blood chimeric equilibrium within 7 to 21 days. Thirty-three parabionts were created between age matched wild type and GFP+ transgenic mice and analyzed at various time-points thereafter ranging from 12-54 weeks post surgery. Although few (n=0-2) cerebellar heterokaryons were detected in parabionts after 12 weeks as parabionts (data not shown), a significant number (n=12-43) were detected after 20-26 weeks as parabionts. (Fig. 2b). All heterokaryons were binucleated and expressed the red Purkinje neuron-marker Calbindin. These data demonstrate that formation of cerebellar heterokaryons occurs in the absence of variables associated with the BMT procedure. Thus, HSCs not introduced by cell transplantation can fuse with Purkinje neurons in the absence of lethal irradiation.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: 10/30/07 Signature: _____
Helen Blau, Ph.D.

Figure 1.

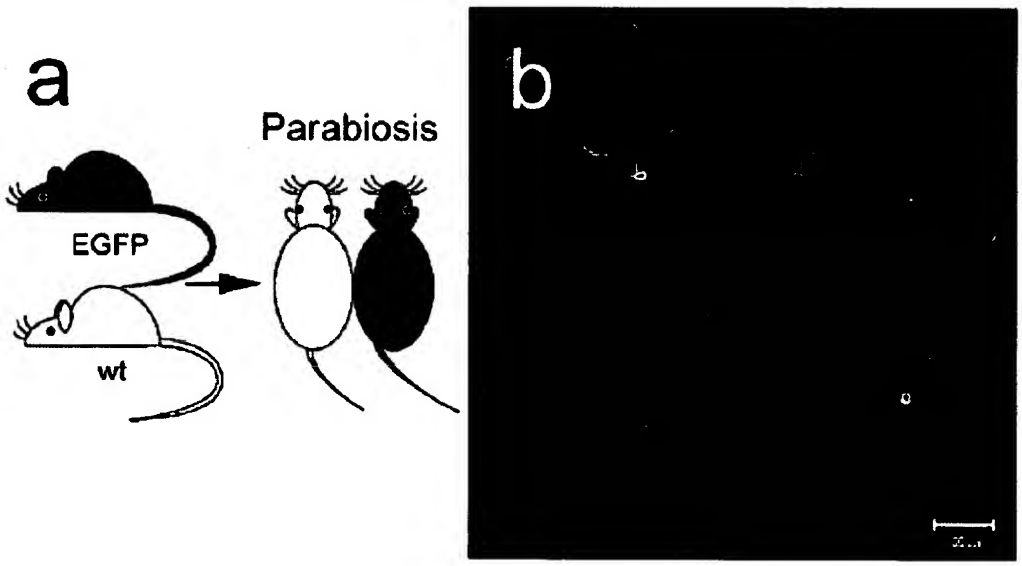


Mice transplanted with a single HSC

Mouse Id	Time after Transplant.	Blood Chimerism (%)	Heterokaryons (n)
#1		>30	2
#2		>30	1

Figure 1. Progeny of a single HSC can form heterokaryons with Purkinje neurons. (a) Schematic drawing of the experimental design. Bone marrow (BM) from EGFP mice is harvested and the side population (SP) is further enriched for HSCs and FACS sorted for single HSCs. A single HSC is transplanted with 10^6 helper cells into a lethally irradiated wild-type mouse. (b) A high power laser-scanning confocal image from a cerebellar sagittal section of a mouse transplanted with a single HSC. Immunohistochemistry with specific antibodies demonstrates the presence of GFP in a single cerebellar heterokaryon (green) co-expressing Calbindin (red) in Purkinje neurons. The GFP^+ Purkinje neuron is located in the Purkinje cell layer (PCL) with dendrites extending into the molecular layer (ML). Whereas the only output connection from the cerebellum to the cerebellar nucleus is mediated by a single axon (arrow) descending through the granular cell layer (GCL).

Figure 2.



Parabionts without Idiopathic Ulcerative Dermatitis

Mouse Id	Time as Parabionts (w)	Blood Chimerism (%)	Heterokaryons (n)
#15	20	50	**12**
#16	20	56	**24**
#11	26	55	**43**
#12	26	57	**16**

Figure 2. Fusion and formation of cerebellar heterokaryons is independent of variables associated with bone marrow transplantation. (a) Schematic drawing of the experimental design of surgical joining of mice to form parabionts. Two female mice (one wild type, the recipient and one transgenic mouse, ubiquitously expressing EGFP, the donor) are surgically joined from the olecranon to the knee. (b) Immunohistochemistry with cell-specific antibodies demonstrates the presence of single GFP+ Purkinje neurons (green), heterokareons, a product of a GFP^+ BMDC and a Purkinje neuron, co-expressing Calbindin (red). The reconstitution of the peripheral blood was around 50% and the parabionts survived 20 to 26 weeks post-surgery. No gross pathology was detected in these mice such as dermatitis.

Effects of Recombinant Human Granulocyte Colony-Stimulating Factor on Hematopoietic Progenitor Cells in Cancer Patients

By Ulrich Dührsen, Jean-Luc Villeval, Janis Boyd, George Kannourakis, George Morstyn, and Donald Metcalf

Hematopoietic progenitor cell levels were monitored in the peripheral blood and bone marrow of 30 cancer patients receiving recombinant human granulocyte-colony stimulating-factor (rG-CSF) in a phase I/II clinical trial. The absolute number of circulating progenitor cells of granulocyte-macrophage, erythroid, and megakaryocyte lineages showed a dose-related increase up to 100-fold after four days of treatment with rG-CSF and often remained elevated two days after the cessation of therapy. The relative frequency of different types of progenitor cells in peripheral blood remained unchanged. The frequency of progenitor cells in the marrow was variable after rG-CSF treatment but in most patients was slightly decreased. The responsiveness of bone marrow progenitor cells to stimulation in vitro by rG-CSF and granulocyte-macrophage colony-stimulating factor did not change significantly during rG-CSF treatment. In patients nine days after treatment with melphalan and then rG-CSF, progenitor cell levels were very low with doses of rG-CSF at or below 10 μg/kg/d, but equaled or exceeded pretreatment values when 30 or 60 μg/kg/d of rG-CSF was given.

GRANULOCYTE COLONY-stimulating factor (G-CSF) is a proliferative stimulus for precursors of neutrophils in vitro.[1,2] Human G-CSF has been purified from various sources,[3-5] cDNAs cloned and expressed in bacterial[6] and mammalian cells,[7] and the bacterially synthesized recombinant molecule (rG-CSF) found to have the same range of activities in vitro as the native hormone.[6]

Investigations in animals have indicated that G-CSF is capable of inducing a significant increase in total granulocytes both in healthy animals[8-10] and in animals with congenital[11] or drug-induced defects of the granulocyte-macrophage system,[12-14] thereby leading to enhanced resistance to microbial infections.[15] Recent clinical trials with cancer patients receiving cytotoxic chemotherapy have shown that the same effects of G-CSF can be observed in humans.[16-19]

G-CSF is a proliferative stimulus for granulocyte progenitor cells, but these are transit cells with no capacity for self-renewal. Administration of G-CSF in vivo might therefore deplete progenitor cell numbers unless other mechanisms compensate for the depletion. To monitor these possible effects of G-CSF, we analyzed progenitor cell levels in the peripheral blood and bone marrow of patients who were treated with rG-CSF in a phase I/II clinical trial reported elsewhere.[17,18]

From the Cancer Research Unit, The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital; the Clinical Research Programme, Melbourne Tumour Biology Branch, Ludwig Institute for Cancer Research, Royal Melbourne Hospital; and the Department of Clinical Haematology and Oncology, Royal Children's Hospital, Victoria, Australia.

Submitted April 25, 1988; accepted August 22, 1988.

Supported in part by the Deutsche Forschungsgemeinschaft (Grant Du 152/1-1), the Institut National de la Santé et de la Recherche Médicale, the Carden Fellowship Fund of the Anti-Cancer Council of Victoria, and the National Health and Medical Research Council, Canberra, Australia.

Address reprint requests to Ulrich Dührsen, MD, Cancer Research Unit, The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital PO 3050, Victoria, Australia.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/88/7206/0110$3.00/0

MATERIALS AND METHODS

Criteria for Eligibility and Description of Patients

The criteria for patient eligibility were reported in detail elsewhere.[17,18] In brief, patients had histologic evidence of metastatic cancer with a life expectancy of at least 2 months. No chemotherapy or radiotherapy had been administered in the 6 weeks preceding treatment with rG-CSF, and radiotherapy was limited to less than 50% of the bone marrow. The type of tumor had to justify treatment with melphalan, and significant nonmalignant disease such as cardiac, respiratory, renal, or hepatic dysfunction was excluded. Pretreatment hemoglobin levels were greater than 10 g/dL, polymorphonuclear cell counts greater than 1,500/μL, and platelet counts greater than 100,000/μL. Before initiation of treatment the patients gave informed consent.

The study was performed at the Royal Melbourne Hospital under the ethical guidelines of the National Health and Medical Research Council of Australia and the Food and Drug Administration of the United States and comprised a total of 30 patients who fulfilled the aforementioned requirements (Table 1).

Properties of rG-CSF

rG-CSF was supplied by Amgen Corp, Thousand Oaks, CA. The 174-amino acid protein is a nonglycosylated single-chain polypeptide with a molecular weight of about 18.6 kilodaltons that is produced by *Escherichia coli* and purified via a series of chromatographic steps.[6] The final product was formulated in an aqueous buffer as a sterile solution at a concentration of 0.25 mg/mL, and each batch was demonstrated to be biologically active and free from pyrogens before release.

Study Protocol and Drug Dosage

Each patient received two cycles of rG-CSF. The first cycle consisted of a five-day treatment period with rG-CSF alone followed by a therapy-free interval of three days' duration. The second cycle aimed at investigating the effects of rG-CSF during drug-induced myelosuppression and began on the fourth interval day. After a single intravenous (IV) injection of the alkylating agent melphalan (25 mg/m^2), rG-CSF treatment continued for nine days at the same dose level as that used in the first cycle.

Administration of rG-CSF was by either the subcutaneous (SC) or IV route, and doses varied from 0.3 to 60 μg/kg body weight/d. In general, groups of three patients were treated with each dose and route of administration. In individual patients the dose of rG-CSF was not varied. For doses of 0.3 and 1 μg/kg, the SC administration

C91

Table 1. Patient Characteristics and Dosage of rG-CSF

Daily Dose of rG-CSF	SC Administration				IV Administration			
	Age, Sex	Origin and Type of Tumor	CT	RT	Age, Sex	Origin and Type of Tumor	CT	RT
0.3 μg/kg	63, M	Rectum (adeno-Ca)	+	−				
	66, M	Pleura (undifferentiated)	+	−				
	66, F	Lung (undifferentiated Ca)	+	−				
1 μg/kg	58, M	Lung (squamous cell Ca)	+	+	68, M	Lung (small cell Ca)	+	+
	60, M	Oropharynx (squamous cell Ca)	+	+	51, M	Lung (adeno-Ca)	−	−
	57, M	Lung (squamous cell Ca)	−	−	64, F	Kidney (Ca)	−	−
3 μg/kg	74, M	Lung (squamous cell Ca)	−	−	66, F	Ovary (adeno-Ca)	+	+
	78, F	Unidentified (adeno-Ca)	−	−	60, M	Colon (adeno-Ca)	−	−
	61, M	Rectum (adeno-Ca)	+	−	69, F	Bone marrow (multiple myeloma)	+	+
	64, M	Colon (adeno-Ca)	+	−				
	46, M	Lung (small cell Ca)	+	+				
	41, M	Lymph node (non-Hodgkin's lymphoma)	+	+				
10 μg/kg	64, F	Abdomen (carcinoid)	−	−	62, F	Stomach (adeno-Ca)	−	−
	70, M	Lymph node (non-Hodgkin's lymphoma)	+	−	56, M	Unidentified (adeno-Ca)	−	−
	68, M	Lung (adeno-Ca)	−	−	75, M	Parotid (adeno-Ca)	−	+
30 μg/kg					62, F	Unidentified (adeno-Ca)	−	−
					61, M	Bone marrow (multiple myeloma)	+	+
					66, M	Lung (adeno-Ca)	−	−
60 μg/kg					69, M	Colon (adeno-Ca)	−	−
					45, F	Unidentified (undifferentiated)	+	−
					46, M	Rectum (adeno-Ca)	−	+

Abbreviations: CT, previous chemotherapy; RT, previous radiotherapy; Ca, carcinoma.

consisted of a single daily injection, and 10 μg/kg was administered as a continuous SC infusion over the entire treatment period by using Cormed infusion pumps (Medical Specialities, East Kew, Victoria, Australia). The intermediate dose of 3 μg/kg was administered either as an SC bolus injection or as a continuous infusion. For IV administration, the daily amount of rG-CSF was divided into two doses administered at 12-hour intervals in a volume of 100 mL of 5% glucose as a 20- to 30-minute infusion via a central venous line. Doses administered IV ranged from 1 to 60 μg/kg/d, thus allowing a comparison with the SC administration at the 1-, 3-, and 10-μg/kg levels (Table 1).

Collection and Processing of Blood and Bone Marrow Samples

Peripheral blood was obtained on the day preceding the first treatment cycle, after four days of rG-CSF treatment, two days after the end of the first cycle, and on the last day of the second rG-CSF course, ie, nine days after the administration of melphalan. To minimize possible interference of accessory cells with colony formation by progenitor cells,[20] samples were fractionated by density gradient centrifugation and removal of adherent cells before culture. An aliquot of the blood sample was used to obtain a full blood count, and the remaining volume was subjected to gradient centrifugation (30 minutes at 400 g) using Ficoll-Hypaque (density, 1.077 g/mL; Pharmacia, Inc, Uppsala, Sweden). This fractionation step was routinely monitored by absolute cell counts and cytocentrifuge preparations of cells from the interface and pellet that were stained with May-Grünwald-Giemsa and then typed morphologically. The interface mononuclear cell fraction was washed, suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), and incubated in 90-mm plastic Petri dishes for one hour at 37°C to remove adherent cells.[20] The proportion of nonadherent cells was determined for each specimen from cell counts before and after depletion of adherent cells and the nonadherent cell fraction used for progenitor cell assays.

Bone marrow specimens were obtained by sternal aspiration on the day before initiation of the first rG-CSF cycle and after four days of treatment. Gradient centrifugation and depletion of adherent cells were performed as described for peripheral blood cells.

Progenitor Cell Assays

All progenitor cell assays were performed in 1-mL cultures in 35-mm plastic Petri dishes. Peripheral blood cells were usually cultured at 2×10^5 cells/dish and bone marrow cells at 5×10^4 cells/dish. The cultures were incubated in a fully humidified atmosphere of 5% CO_2 in air at 37°C for up to 14 days.

Granulocyte-macrophage and early erythroid progenitor cells were grown in agar cultures prepared by adding 1 vol of double-strength Iscove's modified Dulbecco's medium and 1 vol of FCS to 2 vol of 0.6% agar.[1] Granulocyte-macrophage progenitor cells were stimulated by the addition to each dish of (*a*) 500 units of purified rG-CSF (specific activity, 2×10^7 U/mg) dissolved in a volume of 0.1 mL of saline, (*b*) 1,500 units of purified recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (specific activity, 2×10^7 U/mg), (*c*) a mixture of 500 units of rG-CSF plus 1,500 units of rGM-CSF, or (*d*) 0.1 mL of normal saline for the detection of spontaneous colony development. (Fifty units per milliliter is defined as the concentration of a CSF stimulating the formation of half-maximal numbers of colonies in conventional agar cultures of bone marrow cells.[1]) Since the responsiveness to GM-CSF of bone marrow cells from different individuals varied widely (see Results), a higher concentration of GM-CSF was used to ensure maximal stimulation of progenitor cells. For each stimulus, cultures were prepared in quadruplicate and duplicate dishes scored routinely after 5, 7, and 14 days of incubation by using a dissection microscope at 35× magnification. Clones of three to 40 cells were scored as clusters and clones of more than 40 cells as colonies.[1] Cultures were then fixed with 2.5% glutaraldehyde, transferred to glass slides, and stained with Luxol–fast blue and hematoxylin for differential colony counts.[1]

Early erythroid progenitor cells were stimulated by 2 units of recombinant erythropoietin (rEpo, supplied by Amgen) plus 0.1 mL of a preparation of human placental conditioned medium (HPCM) to stimulate the development of maximal numbers of colonies.[21] Cultures were scored after 14 days of incubation and then fixed and stained for differential colony counts as before. Discrimination between pure erythroid and mixed erythroid colonies was based on nuclear morphology.

Late erythroid progenitor cells (colony-forming units, erythroid; CFU-E) were assayed in methylcellulose cultures by using 2 U/mL of rEpo.[1] The cultures were scored after seven days of incubation by using an inverted microscope and CFU-E identified as hemoglobinized clones of eight cells or more.

Megakaryocytic colonies were grown by a plasma clot technique using 4×10^5 peripheral blood cells or 2×10^5 bone marrow cells per 1-mL dish.[22] After 11 days of incubation the cultures were dried, labeled with a monoclonal mouse antibody against the glycoprotein IIb/IIIa complex (antibody WM 18, kindly provided by Dr M. Berndt, Sydney, Australia),[23] and stained with a fluorescein-conjugated sheep antimouse immunoglobulin antibody (Silenus Laboratories, Hawthorn, Victoria, Australia). Megakaryocyte colonies were identified under the fluorescence microscope as clones of three cells or more with intense surface labeling.

Calculation of Progenitor Cell Numbers per Blood Volume

In most of the data, levels of progenitor cells are expressed as the number per 10^5 nonadherent mononuclear cells. To calculate the rise in absolute numbers of progenitor cells induced by rG-CSF treatment (see Fig 4), the numbers of progenitor cells were corrected for changes in total mononuclear cells determined on the basis of white cell differential counts and changes in the ratio between adherent and nonadherent cells. With the exception of one patient (see Results), the cell fractions from the interface were comparable in mononuclear cell composition before and after treatment with rG-CSF. The changes in the absolute numbers of mature granulocytes and granulocyte-macrophage progenitor cells (see Fig 4) were expressed as the ratio between the absolute numbers per unit blood volume by comparing samples from individual patients before and after treatment.

Statistical Analysis

The *t* test for paired samples (one tailed) was used to compare progenitor cell numbers before and after treatment with rG-CSF.

RESULTS

Fractionation of Cell Samples

Before therapy with rG-CSF, the fraction of peripheral blood cells collected from the interface of the Ficoll gradient consisted of 95.8% ± 4.0% (range, 87% to 100%) mononuclear cells composed almost exclusively of cells with lymphocytelike or monocyte morphology and less than 5% contaminating granulocytes. In blood from patients injected with up to 30 μg/kg/d of rG-CSF, the percentage of mononuclear cells in this fraction remained virtually unchanged (after four days of rG-CSF therapy, 94.0% ± 6.8% [83% to 100%]; two days after the cessation of therapy, 96.8% ± 3.8% [88% to 100%]), although in some patients a small proportion of immature granulocytic precursors was observed in addition to the dominant lymphocytelike cells and monocytes. In blood from patients administered 60 μg/kg/d of rG-CSF, the fractionated samples from two patients exhibited the same composition as that observed with lower rG-CSF doses, but the low-density cell fraction collected from the third patient during and after rG-CSF therapy unexpectedly contained 72% to 75% mature granulocytes, band forms, and metamyelocytes. In this case, the frequencies of progenitor cells were corrected according to the actual numbers of mononuclear cells cultured.

The composition of the high-density cell fraction sampled from the pellet of the density gradient did not change significantly during therapy with rG-CSF. Neutrophilic and eosinophilic granulocytes were the major cell types (before rG-CSF treatment, 93.3% ± 7.0%; after four days of therapy, 94.1% ± 5.5%; two days after the end of treatment, 95.3% ± 3.7%), with only a minority of contaminating mononuclear cells.

Treatment with rG-CSF had no consistent influence on the ratio between adherent and nonadherent cells within the mononuclear cell fraction. In peripheral blood, the proportion of nonadherent cells was 74% ± 19% (range, 39% to 100%) before therapy as compared with 66% ± 24% (25% to 100%) during and 72% ± 23% (16% to 100%) after treatment with rG-CSF. In bone marrow, the respective pretreatment and posttreatment values were 81% ± 18% (39% to 100%) and 84% ± 15% (50% to 100%). The fluctuations in the percentage of adherent cells in samples collected at different times from the same patient did not generally exceed a two- to threefold difference, and any variability observed was not correlated with the dose of rG-CSF administered.

Frequencies of Blood Progenitor Cells

Early progenitor cells. The frequency of circulating day 14 colony-forming cells (CFCs) (presumptively less mature progenitors) responsive to stimulation by a mixture of rG-CSF and rGM-CSF rose significantly ($P < .01$) during treatment with rG-CSF (Fig 1). In most patients, this effect was still demonstrable two days after discontinuation of rG-CSF treatment. In cultures stimulated by either rG-CSF or rGM-CSF, the levels of G-CSF–responsive and GM-CSF–responsive progenitor cells both rose to a similar extent following rG-CSF therapy. With all doses of rG-CSF administered, granulocyte, granulocyte-macrophage, macrophage, and eosinophil CFCs showed, overall, no significant change in relative frequencies (Fig 2).

Megakaryocyte colony formation was monitored with cells from three patients treated with 3 or 10 μg/kg/d of rG-CSF SC or 30 μg/kg/d IV, respectively. In the patient receiving the lower SC dose, circulating megakaryocyte CFCs rose from 1.5 per 10^5 mononuclear nonadherent cells before therapy to 8.5 during rG-CSF administration and 9.5 after the cessation of therapy. The respective values in the other SC-treated patient were 7, 66.5, and 41.5. In the patient receiving rG-CSF IV, treatment induced a rise in the frequency of megakaryocyte progenitor cells from 4.5 to 26 per 10^5 cells (no samples were analyzed after withdrawal of rG-CSF therapy).

Cultures stimulated by rEpo plus HPCM consistently showed a comparable rise in the number of hemoglobinized day 14 CFCs following the administration of rG-CSF.

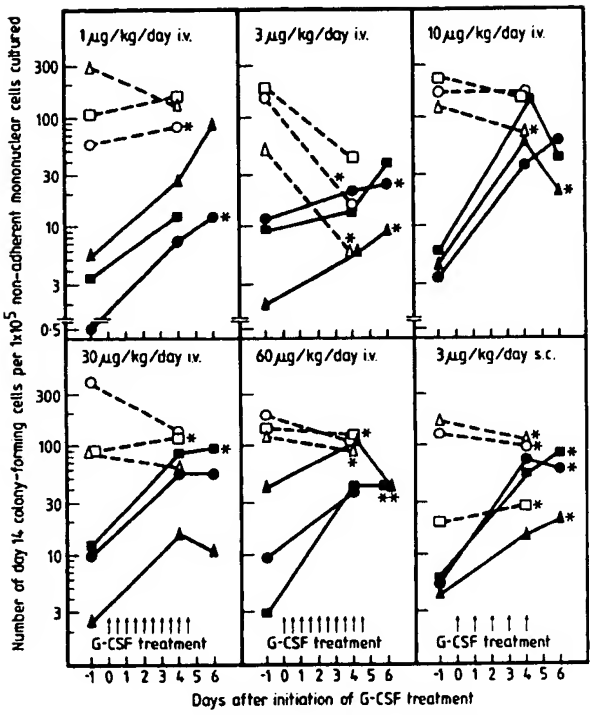


Fig 1. Frequency of day 14 CFCs in peripheral blood (closed symbols, solid lines) and bone marrow (open symbols, broken lines) before, during, and after treatment with different doses of rG-CSF. Each point represents mean values from duplicate agar cultures stimulated by 500 units of rG-CSF plus 1,500 units of rGM-CSF. Each panel contains data from three patients (circles, squares, triangles) treated at the same dose level of rG-CSF. Asterisks denote patients with previous chemotherapy and/or radiotherapy.

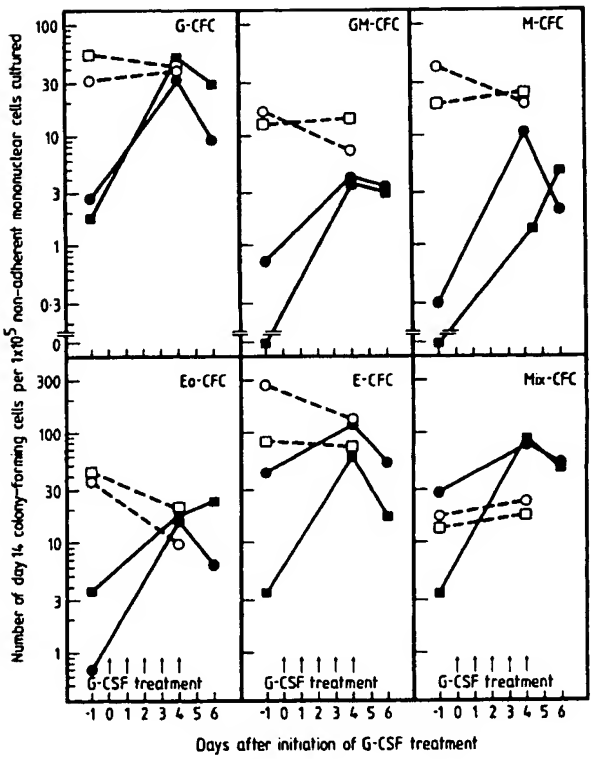


Fig 2. Frequency of different types of day 14 CFCs in peripheral blood (closed symbols, solid lines) and bone marrow (open symbols, broken lines) from two patients before, during, and after treatment with rG-CSF. Circles, data from a patient treated with 10 μg/kg/d of rG-CSF IV; squares, data from a patient treated with 3 μg/kg/d SC. Abbreviations: G-CFC, granulocyte colony-forming cells; GM, granulocyte-macrophage; M, macrophage; Eo, eosinophil; E, pure erythroid; Mix, mixed erythroid. G-, GM-, M-, and Eo-CFCs were enumerated in cultures stimulated by 500 units of rG-CSF plus 1,500 units of rGM-CSF, and E- and Mix-CFCs were enumerated in cultures stimulated by 2 units of rEpo plus 10% HPCM.

Differential colony counts indicated that the increase affected both pure erythroid and mixed erythroid colonies (Fig 2). These latter colonies did not vary significantly in morphology before and after treatment with rG-CSF and contained predominantly granulocytic and less frequently macrophage or eosinophilic elements in addition to erythroid cells. The characteristic feature of erythroid progenitors in blood, with a higher proportion of mixed colonies than in cultures of marrow, did not change in the major increase following treatment with rG-CSF.

The increased levels of progenitor cells detected in peripheral blood after therapy with rG-CSF might have been due either to increased numbers of circulating CFCs or an altered proportion of accessory cells with inhibitory or enhancing effects on colony formation in vitro. To exclude the latter possibility, mixing experiments were performed by using pretreatment and posttreatment blood samples from a patient treated with 10 μg/kg/d of rG-CSF SC and mononuclear nonadherent blood cells from an untreated healthy donor. Before, during, and after treatment with rG-CSF the colony numbers in the combined cultures did not deviate significantly from the sum of the numbers of colonies developing in cultures prepared from each population alone.

Late progenitor cells. Clusters stimulated by G-CSF and scored after five days of incubation (day 5 clones) primarily are formed by promyelocytes and myelocytes, and this parameter can be used to assess the number of late precursor cells.[24,25] In bone marrow cultures the frequency of colonies at day 14 was only 6.3% ± 3.9% (analysis of 98 cultures) that of clusters at day 5. In contrast, in cultures of peripheral blood cells the ratio between day 14 CFCs and day 5 clones was 40.9% ± 28.4% (analysis of 168 cultures), which suggests that a significant fraction of the day 5 clones in fact later developed into day 14 colonies. The ratio between day 14 CFCs and day 5 clones in blood cultures was not changed significantly by treatment with rG-CSF. In the erythroid lineage, colony size, cell size, and hemoglobinization after only seven days of incubation were used as parameters to identify late progenitor cells (CFU-E).

Late granulocyte and erythroid progenitors both showed a remarkable increase in numbers following rG-CSF administration (Fig 3). The presence of significant numbers of CFU-E was particularly striking since this type of progenitor cell was usually undetectable in the blood before treatment. These peripheral blood CFU-E were usually larger and frequently less intensely hemoglobinized than were the CFU-

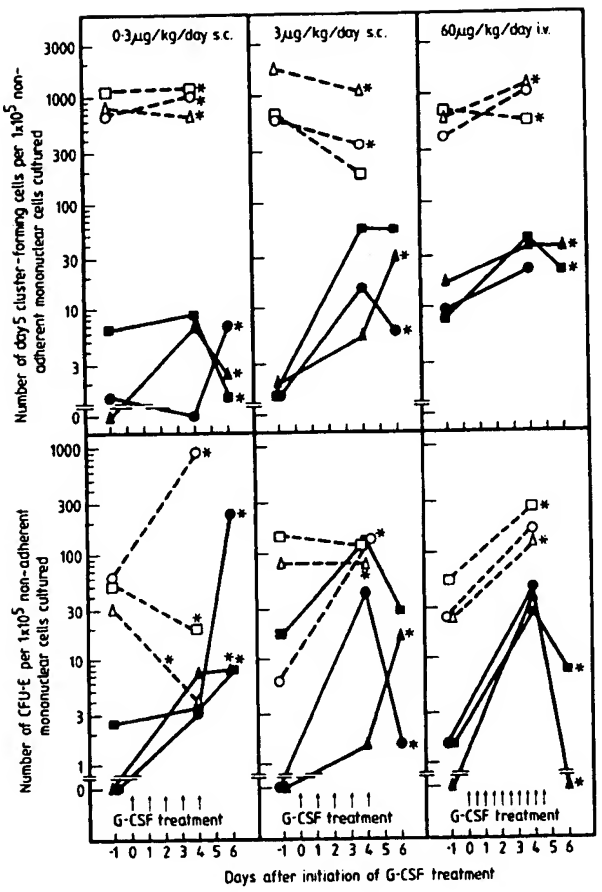


Fig 3. Frequency of day 5 cluster-forming cells (top panels) and CFU-E (bottom panels) in peripheral blood (closed symbols, solid lines) and bone marrow (open symbols, broken lines) before, during, and after treatment with different doses of rG-CSF. Each panel contains data from three patients (circles, squares, triangles) treated at the same dose level of rG-CSF. Asterisks denote patients with previous chemotherapy and/or radiotherapy.

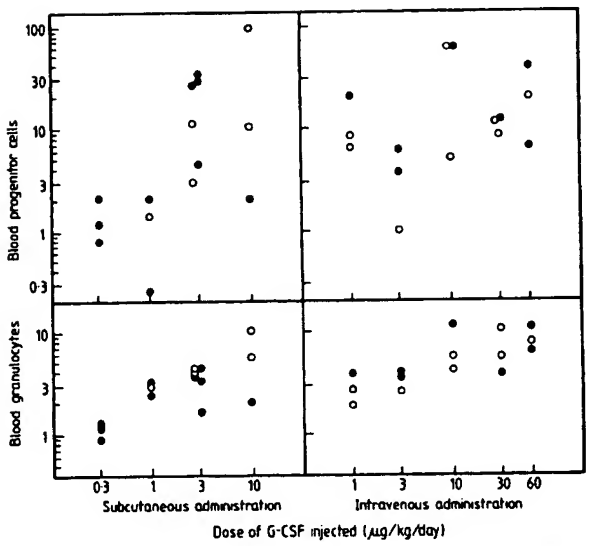


Fig 4. Dose-related changes in the ratio (four days after rG-CSF treatment *v* pretreatment) of blood progenitor cells (top panels) and blood neutrophils (bottom panels) induced by treatment with rG-CSF. Open circles, patients with no history of previous chemotherapy and/or radiotherapy; closed circles, patients with previous chemotherapy and/or radiotherapy. Left panels, data from patients receiving SC bolus injections (0.3 to 3 μg/kg/d) or continuous SC infusions of rG-CSF (3 to 10 μg/kg/d). Absolute numbers of progenitor cells before rG-CSF therapy were 6.6 ± 4.3 × 10^4/L of blood (means and SD) and absolute numbers of neutrophils, 6.1 ± 3.1 × 10^9/L of blood. Right panel, data from patients receiving IV infusions. Pretreatment values for progenitor cells were 9.8 ± 10.1 × 10^4 and for neutrophils, 5.4 ± 3.0 × 10^9/L. Progenitor cells were enumerated in cultures stimulated by 500 units of rG-CSF plus 1,500 units of rGM-CSF after 14 days of incubation.

E developing in bone marrow cultures, thus suggesting a slightly earlier maturational stage.

Dose-response relationship. The administration of rG-CSF was followed by a dose-dependent increase in the numbers of neutrophilic granulocytes in peripheral blood.[17,18] We compared the effect of individual doses of rG-CSF on mature neutrophils with that on day 14 CFCs stimulated by a combination of rG-CSF and rGM-CSF.

Figure 4 demonstrates that the capacity of rG-CSF to elevate the levels of mature blood granulocytes was dose related. Both for the SC and the IV routes of delivery, maximal effects were observed at 10 μg/kg/d of rG-CSF, with no further increase at higher IV doses. Before treatment with rG-CSF the average calculated number of circulating day 14 CFCs was 8.2 ± 7.7 × 10^4/L of blood. For the SC route of administration the increase in calculated total blood progenitor cell numbers showed a similar dose dependency to the rise in mature neutrophils, pronounced increases being observed at the 3- and 10-μg/kg dose levels ($P < .01$). In contrast, no clear-cut dose-response relationship was observed after IV infusion of rG-CSF, and the lowest doses of IV administered rG-CSF proved efficient in inducing a considerable increase in the numbers of circulating CFCs ($P < .01$). While the maximal rise in neutrophil numbers did not exceed an 11-fold increase after four days of rG-CSF therapy, blood progenitor cell numbers increased up to 100-fold. In patients treated with 10 μg/kg/d of rG-CSF or higher, the calculated numbers of blood progenitor cells ranged from 1 to 5 × 10^6/L.

Circulating erythroid and megakaryocyte progenitor cells showed the same increase in number as their counterparts in the granulocyte-macrophage lineage. In contrast, the numbers of mature erythrocytes and platelets did not change significantly during rG-CSF therapy.[17,18]

Colony formation in unstimulated cultures. Unstimulated agar cultures prepared from blood cells before the initiation of rG-CSF therapy frequently showed the formation of low numbers of clusters (usually macrophage) and colonies (mainly granulocytic). In blood cultures from some patients treated with high doses of rG-CSF SC or IV (10 to 60 μg/kg/d) the frequency of spontaneously developing colonies increased five- to 20-fold after therapy, but again granulocytic colonies were by far the most frequent colony type.

Frequencies of Bone Marrow Progenitor Cells

In contrast to the consistent changes in the number of peripheral blood CFCs, the behavior of the marrow progenitor cells was more variable. In 70% of patients, treatment with rG-CSF was associated with a decrease in the number of day 14 CFCs, which affected both the granulocyte-macrophage and erythroid lineages (Figs 1 to 3). Likewise, megakaryocyte progenitor cell levels dropped to 30% or 67% of pretreatment values in two patients receiving 10 or 30 μg/kg/d of rG-CSF, respectively. There was no consistent correlation between a history of prior cytotoxic treatment or irradiation and the observed reduction in the frequency of bone marrow CFCs after rG-CSF treatment (Fig 1). While late granulocyte-macrophage progenitor cells tended to show the same changes during rG-CSF therapy as their more immature precursors (decrease in number in 74% of patients), an increase in frequency was more common for CFU-E (61% of patients, Fig 3). Spontaneous colony and cluster formation in unstimulated bone marrow cultures did not change noticeably during treatment with rG-CSF.

In Vitro Responsiveness of Progenitor Cells to rG-CSF and rGM-CSF

To investigate whether treatment with rG-CSF induced changes in the responsiveness of progenitor cells to CSFs, bone marrow cells obtained from six patients before and after four days of rG-CSF treatment were stimulated in agar cultures with serial twofold dilutions of known amounts of rG-CSF or rGM-CSF. The CSF concentrations stimulating the development of half-maximal numbers of colonies were determined graphically (effective concentration, 50% [EC_{50}]) and used to characterize the responsiveness of the marrow cells.

Untreated bone marrow cells from different patients showed little variation in responsiveness to rG-CSF, which is illustrated by similar EC_{50} values before rG-CSF therapy (Table 2). In contrast, responsiveness to rGM-CSF varied widely from patient to patient, in general associated with a more shallow CSF titration curve. In individual patients, however, the EC_{50} values both for rG-CSF and rGM-CSF were in the same range before and after the administration of rG-CSF, which indicates that a significant change in the responsiveness of the progenitor cells to the CSFs had not occurred (Table 2). In addition, the shape of the dose-response curves remained uninfluenced by rG-CSF therapy.

Frequencies of Blood Progenitor Cells During Drug-Induced Myelosuppression

Nine days after melphalan treatment, patients receiving 0.3 to 3 μg rG-CSF/kg/d had no detectable progenitors in the peripheral blood. In patients receiving 10 μg rG-CSF/kg/d or higher, progenitor cells were detectable, and with doses of 30 and 60 μg rG-CSF/kg/d, progenitor cell numbers equaled or slightly exceeded those in the same patients before initiation of the first treatment cycle with rG-CSF.

Table 2. Concentrations in Units of rG-CSF and rGM-CSF Stimulating the Development of Half-Maximal Numbers of Colonies (EC_{50}) in Cultures of Bone Marrow Cells Obtained From Six Patients Before and After Treatment With Different Doses of rG-CSF

Daily Dose of rG-CSF	EC_{50} (U/mL) for Stimulation With rG-CSF		EC_{50} (U/mL) for Stimulation With rGM-CSF	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
0.3 μg/kg SC	91	99	181	198
1 μg/kg IV	42	62	9	5
	91	69	94	152
3 μg/kg SC	31	33	12	9
30 μg/kg IV	27	20	2	5
	18	6	2	4

Data on each line refer to results when using marrow cells from a different patient. Serial twofold dilutions of known amounts of rG-CSF and rGM-CSF were used to stimulate agar cultures containing 5×10^4 nonadherent bone marrow cells from patients before and after four days of treatment with rG-CSF. Cultures stimulated by rG-CSF were scored after 7 days and cultures stimulated by rGM-CSF after 14 days of incubation. Fifty units per milliliter is the concentration of CSF required for half-maximal stimulation of normal human marrow cells.

DISCUSSION

On the basis of the known actions of G-CSF in vitr[1,2,4,6,26] the administration of G-CSF in vivo would be expected to induce an increase in the number of mature granulocytes at the expense of the granulocytic progenitor pool. While G-CSF induced major increases in circulating granulocytes in experimental animals[8-15] and patients receiving chemotherapy,[16-19] investigations in mice unexpectedly showed an increase in the total number of hematopoietic progenitor cells in spleen and bone marrow after G-CSF administration[12,13] that not only included granulocyte-macrophage CFCs but also erythroid and megakaryocyte CFCs and pluripotent stem cells.[12] Assuming that the direct actions of G-CSF in vivo are not different from those in vitro, this observation suggests the interaction of G-CSF with some other regulator or the initiation by G-CSF either of the production of other hematopoietic regulators by accessory cells or some compensatory homeostatic mechanism.

In the patients described here, the administration of rG-CSF was followed by a pronounced increase in the number of circulating progenitor cells and, in most patients, a slight reduction in the frequency of bone marrow progenitor cells. The increase in the absolute numbers of circulating CFCs affected all types and maturational stages of progenitor cells assayed and was already demonstrable at low doses of IV-injected rG-CSF. Although the frequency of CFCs in blood approached the frequency in bone marrow, characteristic features of the relative frequency of progenitor cell subsets in peripheral blood were retained after rG-CSF therapy. Thus, the ratios between day 14 CFCs and day 5 clones, between eosinophil and granulocyte-macrophage colonies, and between mixed erythroid and pure erythroid colonies remained virtually unchanged. This argues against the interpretation that the increased circulating progenitor cells were simply the consequence of unselective release of CFCs from the marrow.

The mechanisms responsible for the rG-CSF–induced increase in peripheral blood progenitor cell numbers require further investigation. Possibilities include the release of

existing marrow progenitor cells, increased production of progenitor cells from stem cells in the marrow or spleen, or a combination of these. Information on the physical nature, surface antigens, and cycling status of these cells would be useful in this context.

Following cytotoxic treatment with alkylating agents, blood CFC numbers usually show a very pronounced initial decrease of 1 week's duration followed by a gradual increase that reaches peak levels 2 to 3 weeks after therapy.[27-29] Our preliminary data suggest that treatment with high doses of rG-CSF may be capable of leading to a more rapid reappearance of progenitor cells in the circulation after chemotherapy, but this needs confirmation by analysis of control patients and a time course analysis.

The significance of the decrease in frequency of bone marrow CFCs is difficult to assess because of a lack of information on total marrow cell counts and the possibility of a variable dilution of marrow aspirates by peripheral blood. Marrow sections from rG-CSF–treated monkeys have demonstrated hypercellularity,[14] thus suggesting that part of the decreased frequency of CFCs that was observed in the present study might be artificial and due simply to an increased proportion of more mature granulocytes rather than to a reduction in the absolute numbers of progenitor cells. In at least some patients after rG-CSF treatment the frequency of CFCs in fractionated blood actually exceeded that in fractionated marrow, so variable dilution by peripheral blood may not account for decreases in these patients.

Successful hematopoietic reconstitution in patients receiving myeloablative treatment has been reported when using stem cells harvested from peripheral blood,[30-35] a procedure that can circumvent the need for general anesthesia and allow the collection of stem cells even if the bone marrow is damaged by previous radiotherapy or infiltrated with malignant cells.[31,36] Furthermore, the neutropenic period following marrow transplantation is possibly reduced after the infusion of stem and progenitor cells derived from peripheral blood.[31] One of the major limitations of the technique is the scarcity of hematopoietic stem cells in the circulation.[33,37] Should the behavior of pluripotent stem cells parallel that of progenitor cells, then injection of donors with rG-CSF might be a useful maneuver before the harvest of peripheral blood cells for transplantation.

ACKNOWLEDGMENT

The authors wish to thank Professor R. Fox, Dr L. Campbell, Sister J. Keech, and the staff of the Haematology Department of the Royal Melbourne Hospital, Australia, for providing blood and marrow specimens used in these studies; Drs L. Souza and K. Alton from Amgen for supplying the rG-CSF and rEpo; L. Di Rago and J. Timpano for excellent technical assistance; Dr M. Berndt for providing antiplatelet antibodies; and Drs G. Johnson, A.W. Burgess, and W. Vainchenker for many helpful discussions.

REFERENCES

1. Metcalf D: The Hemopoietic Colony-Stimulating Factors. New York, Elsevier, 1984

2. Metcalf D, Nicola NA: Proliferative effects of purified granulocyte colony–stimulating factor (G-CSF) on normal mouse hemopoietic cells. J Cell Physiol 116:198, 1983

3. Nicola NA, Begley CG, Metcalf D: Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. Nature 314:625, 1985

4. Welte K, Platzer E, Lu L, Gabrilove JL, Levi E, Mertelsmann R, Moore MAS: Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. Proc Natl Acad Sci USA 82:1526, 1985

5. Nomura H, Imazeki I, Oheda M, Kubota N, Tamura M, Ono M, Ueyama Y, Asano S: Purification and characterization of human granulocyte colony–stimulating factor (G-CSF). EMBO J 5:871, 1986

6. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K: Recombinant human granulocyte colony–stimulating factor: Effects on normal and leukemic myeloid cells. Science 232:61, 1986

7. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H, Ono M: Molecular cloning and expression of cDNA for human granulocyte colony–stimulating factor. Nature 319:415, 1986

8. Metcalf D: The molecular control of normal and leukaemic granulocytes and macrophages.Proc R Soc Lond [Biol] 230:389, 1987

9. Okabe T, Takaku F: In vivo granulocytopoietic activities of human recombinant granulocyte colony–stimulating factor. Exp Hematol 14:475, 1986 (abstr)

10. Cohen AM, Zsebo KM, Inoue H, Hines D, Boone TC, Chazin VR, Tsai L, Ritch T, Souza LM: In vivo stimulation of granulopoiesis by recombinant human granulocyte colony–stimulating factor. Proc Natl Acad Sci USA 84:2484, 1987

11. Lothrop CD Jr, Jones JB, Warren DJ, Moore MAS, Souza LM: Correction of cyclic hematopoiesis with recombinant human granulocyte colony stimulating factor. Blood 70:138, 1987 (suppl 1) (abstr)

12. Tamura M, Hattori K, Nomura H, Oheda M, Kubota N, Imazeki I, Ono M, Ueyama Y, Nagata S, Shirafuji N, Asano S: Induction of neutrophilic granulocytosis in mice by administration of purified human native granulocyte colony–stimulating factor (G-CSF). Biochem Biophys Res Commun 142:454, 1987

13. Shimamura M, Kobayashi Y, Yuo A, Urabe A, Okabe T, Komatsu Y, Itoh S, Takaku F: Effect of human recombinant granulocyte colony–stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood 69:353, 1987

14. Welte K, Bonilla MA, Gillio AP, Boone TC, Potter GK, Gabrilove JL, Moore MAS, O'Reilly RJ, Souza LM: Recombinant human granulocyte colony-stimulating factor. Effects on hematopoiesis in normal and cyclophosphamide-treated primates. J Exp Med 165:941, 1987

15. Matsumoto M, Matsubara S, Matsuno T, Tamura M, Hattori K, Nomura H, Ono M, Yokota T: Protective effect of human granulocyte colony–stimulating factor on microbial infection in neutropenic mice. Infect Immun 55:2715, 1987

16. Bronchud MH, Scarffe JH, Thatcher N, Crowther D, Souza LM, Alton NK, Testa NG, Dexter TM: Phase I/II study of recombinant human granulocyte colony–stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br J Cancer 56:809, 1987

17. Morstyn G, Campbell L, Souza LM, Alton NK, Keech J, Green M, Sheridan W, Metcalf D, Fox R: Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet 1:667, 1988

18. Morstyn G, Campbell L, Dührsen U, Souza LM, Alton NK, Villeval JL, Nicola NA, Boyd AW, Kannourakis G, Cebon J, Thomas R, Boyd J, Keech J, Green M, Sheridan W, Metcalf D, Fox R: Clinical studies with granulocyte colony stimulating factor (G-CSF) in patients receiving cytotoxic chemotherapy, in Proceedings of the CSF Symposium, Behring Institute Mitteilungen No. 83, p 234, 1988

19. Gabrilove JL, Jakubowski A, Scher H, Sternberg C, Wong G, Grous J, Yagoda A, Fain K, Moore MAS, Clarkson B, Oettgen HF, Alton K, Welte K, Souza L: Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N Engl J Med 318:1414, 1988

20. To LB, Haylock DN, Juttner CA, Kimber RJ: The effect of monocytes in the peripheral blood CFU-C assay system. Blood 62:112, 1983

21. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Preparation of colony stimulating factors from human placental conditioned medium. Leuk Res 2:313, 1978

22. Mitjavila MT, Vinci G, Villeval JL, Kieffer N, Henri A, Testa U, Breton-Gorius J, Vainchenker W: Human platelet alpha granules contain a nonspecific inhibitor of megakaryocyte colony formation: Its relationship to type β transforming growth factor (TGF-β). J Cell Physiol 134:93, 1988

23. Berndt MC, Caen JP: Platelet glycoproteins. Prog Hemost Thromb 7:111, 1984

24. Begley CG, Lopez AF, Vadas MA, Metcalf D: The clonal proliferation in vitro of enriched populations of human promyelocytes and myelocytes. Blood 65:951, 1985

25. Begley CG, Metcalf D, Lopez AF, Nicola NA: Fractionated populations of normal human marrow cells respond to both human colony-stimulating factors with granulocyte-macrophage activity. Exp Hematol 13:956, 1985

26. Begley CG, Lopez AF, Nicola NA, Warren DJ, Vadas MA, Sanderson CJ, Metcalf D: Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in vitro: A rapid and sensitive microassay for colony-stimulating factors. Blood 68:162, 1986

27. Richman CM, Weiner RS, Yankee RA: Increase in circulating stem cells following chemotherapy in man. Blood 47:1031, 1976

28. Lohrmann H-P, Schreml W, Fliedner TM, Heimpel H: Reaction of human granulopoiesis to high-dose cyclophosphamide therapy. Blut 38:9, 1979

29. To LB, Haylock DN, Kimber RJ, Juttner CA: High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. Br J Haematol 58:399, 1984

30. Juttner CA, To LB, Haylock DN, Branford A, Kimber RJ: Circulating autologous stem cells collected in very early remission from acute non-lymphoblastic leukaemia produce prompt but incomplete haemopoietic reconstitution after high dose melphalan or supralethal chemoradiotherapy. Br J Haematol 61:739, 1985

31. Körbling M, Dörken B, Ho AD, Pezzuto A, Hunstein W, Fliedner TM: Autologous transplantation of blood-derived hemopoietic stem cells after myeloablative therapy in a patient with Burkitt's lymphoma. Blood 67:529, 1986

32. Reiffers J, Bernard P, David B, Vezon G, Sarrat A, Marit G, Moulinier J, Broustet A: Successful autologous transplantation with peripheral blood hemopoietic cells in a patient with acute leukemia. Exp Hematol 14:312, 1986

33. Tilly H, Bastit D, Lucet J-C, Esperou H, Monconduit M, Piguet H: Haemopoietic reconstitution after autologous peripheral blood stem cell transplantation in acute leukaemia. Lancet 2:154, 1986 (letter)

34. Stiff PJ, Koester AR, Lanzotti VJ: Autologous transplantation using peripheral blood stem cells. Exp Hematol 14:465, 1986 (abstr)

35. Juttner CA, To LB, Haylock DN, Branford A, Dyson P, Kimber RJ: Haemopoietic reconstitution using circulating autologous stem cells collected in very early remission from acute non-lymphoblastic leukaemia. Exp Hematol 14:465, 1986 (abstr)

36. Barr RD, McBride JA: Haemopoietic engraftment with peripheral blood cells in the treatment of malignant disease. Br J Haematol 51:181, 1982

37. McCarthy DM, Goldman JM: Transfusion of circulating stem cells. CRC Crit Rev Clin Lab Sci 20:1, 1984

Exhibit 2

Harvesting and Enrichment of Hematopoietic Progenitor Cells Mobilized Into the Peripheral Blood of Normal Donors by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) or G-CSF: Potential Role in Allogeneic Marrow Transplantation

By Thomas A. Lane, Ping Law, Midori Maruyama, Dennis Young, Julie Burgess, Michael Mullen, Matthew Mealiffe, Leon W.M.M. Terstappen, Alan Hardwick, Maher Moubayed, Fred Oldham, Robert E.T. Corringham, and Anthony D. Ho

To explore the use of stem/progenitor cells from peripheral blood (PB) for allogeneic transplantation, we have studied the mobilization of progenitor cells in normal donors by growth factors. Normal subjects were administered either granulocyte-macrophage colony-stimulating factor (GM-CSF) at 10 μg/kg/d, or G-CSF at 10 μg/kg/d, or a combination of G- and GM-CSF at 5 μg/kg/d each, administered subcutaneously for 4 days, followed by leukapheresis on day 5. Mononuclear cells expressing CD34 (CD34$^+$ cells) were selectively enriched by affinity labeling using Dynal paramagnetic microspheres (Baxter Isolex; Baxter Healthcare Corp, Santa Ana, CA). The baseline CD34$^+$ cells in peripheral blood before mobilization was 0.07% ± 0.05% (1.6 ± 0.7/μL; n = 18). On the fifth day after stimulation (24 hours after the fourth dose), the CD34$^+$ cells were 0.99% ± 0.40% (61 ± 14/μL) for the 8 subjects treated with G-CSF, 0.25% ± 0.25% (3 ± 3/μL, both $P < .01$ v G-CSF) for the 5 subjects administered GM-CSF, and for the 5 subjects treated with G- and GM-CSF, 0.65% ± 0.28% (41 ± 18/μL, $P < .5$ v GM-CSF). Parallel to this increase in CD34$^+$ cells, clonogenic assays showed a corresponding increase in CFU-GM and BFU-E. The total number of CD34$^+$ cells collected from the G-CSF group during a 3-hour apheresis was 119 ± 65 × 10^6 and was not significantly different from that collected from the group treated with G- and GM-CSF (101 ± 35 × 10^6 cells), but both were greater than that from the group treated with GM-CSF (12.6 ± 6.1 × 10^6; $P < .01$ for both comparisons). Analysis of the CD34$^+$ subsets showed that a significantly higher percentage of cells with the CD34$^+$/CD38$^-$ phenotype is found after mobilization with G- and GM-CSF. In the G-CSF group, immunomagnetic selection of CD34$^+$ cells permitted the enrichment of the CD34$^+$ cells in the apheresis product to 81% ± 11%, with a 48% ± 12% yield and to a purity of 77% ± 21% with a 51% ± 15% recovery in the G- and GM-CSF group. T cells were depleted from a mean of 4.5 ± 2.0 × 10^9 to 4.3 ± 5.2 × 10^6 after selection, representing 99.9% depletion. We conclude that it is feasible to collect sufficient numbers of PB progenitor cells from normal donors with one to two leukapheresis procedures for allogeneic transplantation. Subjects treated with the combination of G- and GM-CSF showed an equivalent mobilization of CD34$^+$ cells and CFU-GM as G-CSF alone, and also demonstrated a significantly greater mobilization of cells with the CD34$^+$/CD38$^-$ phenotype.

AUTOLOGOUS TRANSPLANTATION of hematopoietic progenitor cells harvested from the peripheral blood (PB) has been used to reconstitute hematopoiesis after myeloablative chemotherapy.[1-3] The yield of PB progenitor cells (PBPC) collected during leukapheresis is greatly enhanced if harvesting is performed during the early phase of marrow recovery after myelosuppressive chemotherapy,[4-6] or with the combination of chemotherapy and administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF.[7-10] It has also been demonstrated that hematopoietic growth factors alone are capable of mobilizing hematopoietic progenitors into the peripheral circulation.[11-14]

An advantage of autologous transplantation of blood-derived PC compared with marrow-derived cells is the more rapid recovery of granulocytes and platelets after myeloablative high-dose regimens.[3,15-18] This suggests that mobilized PBPC might also shorten the duration of neutropenia in the setting of allogeneic transplantation. A recent study showed the effectiveness of PBPC harvested from normal donors treated with G-CSF in performing syngeneic transplantation.[19] There have also been preliminary reports in which progenitor cells mobilized into the peripheral blood of normal individuals were used to perform or to support allogeneic transplantation.[20-22]

Procurement of a sufficient number of progenitor cells is critical for successful engraftment, and early failures in PBPC transplants may have been caused by an inadequate dose of progenitor cells.[23,24] The optimal timing of PBPC collection has been investigated after chemotherapy in conjunction with growth factors for autologous transplants[25-27]; however, there has been no systematic investigation of the mobilization of hematopoietic progenitor cells by growth factors in normal individuals for the purpose of allogeneic transplantation.[28] The purpose of this study was to determine the feasibility of collecting sufficient PBPC from normal donors for allogeneic transplantation by mobilizing with G-CSF, or GM-CSF, or a combination of both. Because PBPC harvested by apheresis also contain a large number of T

From the Departments of Medicine and Pathology, UCSD School of Medicine, San Diego; Baxter Healthcare Corporation, Immunotherapy Division, Santa Ana; and Becton Dickinson Immunocytometry Systems, San Jose, CA.

Submitted June 10, 1994; accepted September 2, 1994.

Supported in part by University of California, San Diego (UCSD), Cancer Center Foundation; Baxter Healthcare Corporation, Immunotherapy Group, Santa Ana, CA; Cancer Research Coordinating Committee of University of California (UC), Grant No. 6-800575-441775-19900; Academic Senate of UC Award No. RS159M-Ho; Clinical Research Centers Program, Grant No. M01 RR00827, of the National Center for Research Resources, National Institutes of Health.

Address reprint requests to Anthony D. Ho, MD, PhD, UCSD Cancer Center, 200 W Arbor Dr, #8421, San Diego, CA 92103-8421.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/95/8501-0019$3.00/0

CT1

cells, which may increase the risk of graft-versus-host disease (GVHD), we also investigated the performance of a device designed to enrich $CD34^+$ progenitor cells by immunomagnetic separation. Our results show that, using G-CSF or G-CSF combined with GM-CSF, sufficient numbers of progenitor cells can be mobilized and collected from the PB of normal individuals to perform or support allogeneic bone marrow transplantation (BMT) by one to maximally two leukapheresis procedures. The enrichment process also depletes the harvested product of sufficient numbers of T cells to diminish the likelihood of severe GVHD.

MATERIALS AND METHODS

Normal, healthy subjects were recruited using exclusion criteria similar to those used for blood donors.[29] All subjects had a negative serologic test for hepatitis B and C, human immunodeficiency virus, human T-cell lymphotropic virus I/II (HTLV-I/II), and syphilis (RPR) and all had a normal complete blood count, including platelets and leukocyte differential (data not shown). Consent was obtained after careful explanation of the project. Subjects were given either recombinant human (rh) G-CSF (Amgen, Thousand Oaks, CA), or rhGM-CSF (Immunex, Seattle, WA), 10 μg/kg/d, or G-CSF plus GM-CSF, 5 μg/kg/d each, subcutaneously for 4 consecutive days. Injections were given between 8:00 AM and 10:00 AM. The project was reviewed and approved by the UCSD Human Subjects Committee.

A 10-L leukapheresis procedure was performed 24 hours after the fourth dose of cytokine using a Fenwal CS3000 (Deerfield, IL) or COBE Spectra instrument (COBE, Lakewood, CO). Acid-citrate-dextrose (ACD-A; Baxter Healthcare Corp, Santa Ana, CA) was used as the anticoagulant. Cells harvested by leukapheresis were kept at room temperature (22 to 24°C) and transported to the cell processing laboratory within an hour after collection.

PB counts, flow cytometry, and clonogenic assays were performed before the first injection of cytokine, daily for 4 days during growth factor administration, on the day of progenitor cell harvesting, and 24 hours after apheresis. Blood counts were performed in the routine laboratory using an automated cell counter (Coulter, Hialeah, FL) and leukocyte differential counts were performed manually on all blood samples with a leukocyte count greater than 10,000/μL. Mononuclear cells were prepared from EDTA-anticoagulated peripheral blood samples using Percoll (Sigma, St Louis, MO) density gradient separation.

Flow cytometry. Flow cytometry was performed on daily blood samples. Leukocytes in whole blood were stained at room temperature with appropriate antibodies for 20 minutes (fluorescein isothiocyanate- or phycoerythrin-labeled monoclonal antibodies [MoAbs] against CD3, CD5, CD14, CD19, CD33, CD34, CD45, or negative controls). Erythrocytes were lysed and leukocytes fixed with FACS Lyse (Becton Dickinson [BD] Immunocytometry Systems, San Jose, CA) according to instruction of the manufacturer. All MoAbs were obtained from BD unless otherwise noted. Two-color, four-parameter flow cytometric analysis was performed with a FACScan (BD) as previously described.[30,31] Mononuclear cells were gated by forward- and side-scatter signals. Anti-CD45 was used to verify that all of the mononuclear cells were included in this gate. T cells are identified by the expression of CD3 and/or CD5, and B cells by CD19. At least 40,000 events were gated and data were analyzed using Lysis-II software (BD). The stained cell samples were analyzed within 72 hours after preparation.

Analysis of $CD34^+$ subsets was performed on the leukapheresis product before and after enrichment of $CD34^+$ cells, using antibodies to CD38, HLA-DR, CD15, and CD33. For $CD34^+$ cell subsets analysis, the leukapheresis products, and $CD34^+$ cell-enriched samples were lysed with NH_4Cl buffer, washed, the MoAbs added at the appropriate dilutions, then incubated on ice for 30 minutes in the dark.[30,32,33] Cells were washed twice, fixed with phosphate buffer containing 0.5% paraformaldehyde, and stored at 4°C protected from light. Three-color, five-parameter flow cytometric analysis was performed using a FACScan. At least 1×10^6 cells were analyzed and events were recorded in list mode first ungated to quantitate the relative frequencies of major cell types. The samples were reacquired using live gates with forward scatter, side scatter, and CD34 positivity as gating criteria. Data analysis was performed using Paint-a-Gate (BD), which permits transformation of orthogonal light-scattering signals and the identification of cell populations with multidimensional analysis.[31]

Clonogenic assays. Clonogenic assays for granulocyte-macrophage colony-forming unit (CFU-GM), erythroid burst-forming unit (BFU-E), and mixed CFU (CFU-GEMM) were performed as previously described[25] using a methylcellulose assay system.[34] Briefly, 1×10^5 mononuclear cells or 1×10^3 enriched $CD34^+$ cells were aliquoted on duplicate 35-mm dishes containing Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO/BRL, Grand Island, NY) with 1% methylcellulose plates; erythropoietin 2.5 U/mL (Amgen, Thousand Oaks, CA); progenitor cell factor 150 ng/mL (Genzyme, Cambridge, MA); interleukin-3, 150 U/mL (Genetics Institute, Cambridge, MA); and GM-CSF, 50 ng/mL (Immunex, Seattle, WA). The dishes were placed in a humidified 5% CO_2 incubator and clusters of greater than 50 cells were enumerated after 14 days.

Immunomagnetic selection of $CD34^+$ cells. Cells expressing the CD34 antigen[35-37] were enriched from leukapheresis products using the Baxter Isolex system.[38] The principle of this method is as follows: $CD34^+$ cells were enriched by affinity labeling using a murine MoAb (9C5; Baxter Immunotherapy Division, Santa Ana, CA) and subsequently with paramagnetic microspheres coated with goat-antimouse antibodies. The $CD34^+$ cells were then captured by a magnet. The paramagnetic beads were released from the cells by treatment with chymopapain.

The platelet contents in the leukapheresis product were initially reduced by washing in RPMI (GIBCO; Grand Island, NY) containing 1% human serum albumin (HSA; Baxter/Hyland, Glendale, CA) (RPMI/HSA) twice at 200*g* for 10 minutes at ambient temperature. After the final wash, the cells were resuspended in RPMI/HSA containing 0.5% human Ig (HIg) (Baxter/Hyland) or other equivalent Ig preparation. Nucleated cell concentration was measured using a Coulter Counter. The cells were sensitized with an anti-CD34 antibody (9C5; Baxter Immunotherapy) at 0.5 μg/1 $\times 10^6$ cells for 30 minutes at 4°C under slow (~4 rpm) end-over-end rotation. Unbound antibody was removed by washing (three times at 400*g*, 10 minutes in cold), and the cells were resuspended in RPMI/HSA). Sensitized cells were rosetted with Dynal paramagnetic microspheres (coated with sheep-antimouse IgG1 Fc) (Baxter/Fenwal, Deerfield, IL) at 2 cells/bead in a magnetic separation device for 30 minutes at ambient temperature at 1 to 5×10^7 cells/mL of RPMI/HSA/HIG. The device consists of a mixing chamber with integral inlet and outlet tubing for the introduction and removal of cells, a motor rocking/mixing for sensitized cells and beads, primary magnets for the capture of beads/rosettes, and secondary magnet for maximal entrapment of beads after selection and release. A microprocessor was incorporated into the device, which prompted the operator during each step of the selection procedure. After the conclusion of cell/bead mixing, the beads/rosettes were washed three times using 100 mL of RPMI/HSA to remove trapped nontarget cells, and resuspended in 40 mL of RPMI/HSA. Rosetted cells were released from the beads using chymopapain (prepared for Baxter Immunotherapy by Boots Pharmaceutical, Nottingham, UK)[37,38] at 200 picokatal/mL for 15 minutes at room temperature under rocking/mixing, as above. The beads

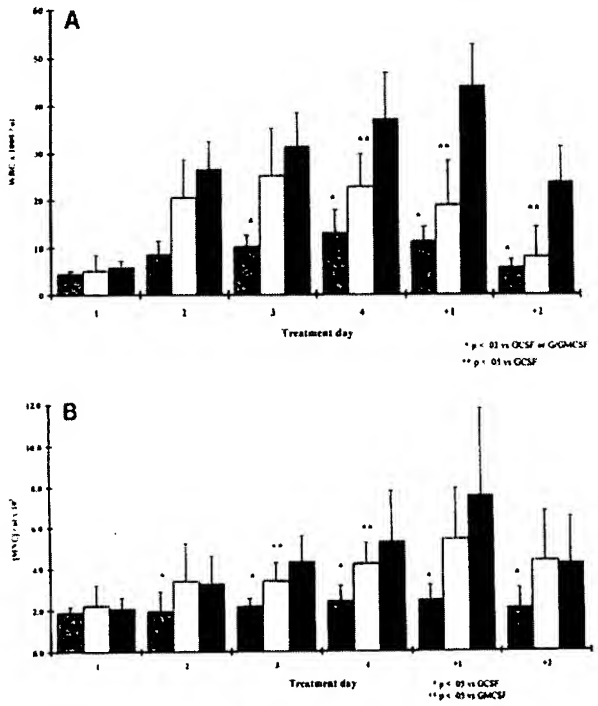


Fig 1. Effect of cytokine administration on the leukocyte count (A) and mononuclear cell count (B). GM-CSF (▨, n = 5), G-CSF + GM-CSF (□, n = 5), or G-CSF (■, n = 8) were administered after blood was drawn on days 1, 2, 3, and 4.

were captured on the primary magnets. The released cells were passed over the secondary magnet to ensure maximal bead capture, washed, and resuspended in RPMI/HSA. Nucleated cell concentration was determined by Coulter Counter and the expression of CD34 and other leukocyte markers by flow cytometry.

Statistical analysis. Data are reported as the mean ± SD unless otherwise noted. Differences between mean values were evaluated for statistical significance by analysis of variance (ANOVA) and a two-tailed unpaired Student's *t*-test, where appropriate, and were calculated on a personal computer using NCSS software (NCSS, Inc. Provo, UT).

RESULTS

Eighteen subjects were studied. Their ages ranged from 21 to 45 years (median age = 28), 8 were men and 10 were women. Eight received G-CSF, five received GM-CSF, and five received the combination of G-CSF and GM-CSF.

Baseline blood counts and leukocyte differentials of all subjects were within normal limits (data not shown). The administration of G-CSF and GM-CSF increased the leukocyte count and number of mononuclear cells in the PB (Fig 1, A and B). Both G-CSF and GM-CSF increased the leukocyte counts of the volunteers; however, G-CSF increased the total leukocyte count as well as the mononuclear cell count significantly more than an equal dose of GM-CSF. Injections of 5 μg/kg each of the combination of G-CSF and GM-CSF gave results intermediate between injections of 10 μg/kg of G-CSF or 10 μg/kg of GM-CSF. No significant change in platelet counts or hemoglobin was observed.

Side effects associated with the injections of cytokines were minor, consisting chiefly of myalgia and fatigue in all subjects. The side effects were mild and were partially or completely ameliorated in all subjects by acetaminophen. All subjects administered G-CSF and GM-CSF completed the full 4 days of therapy and the apheresis procedure. One of four subjects given the combination of G-CSF and GM-CSF (at reduced dosage) elected to discontinue the study because of myalgia and low-grade temperature.

Before cytokine administration the $CD34^+$ cells represented 0.07% ± 0.05% of the peripheral blood mononuclear cells (1.6 ± 0.7 cells/μL; n = 18). This value was not different among the three treatment groups. After administration of the first dose of either G-CSF, GM-CSF, or the combination of G-CSF + GM-CSF there was a progressive increase in the number of $CD34^+$ cells in the PB (Fig 2). These increases were significantly greater in subjects treated with G-CSF or G-CSF + GM-CSF than in those administered GM-CSF alone. There were significant differences in absolute $CD34^+$ cells per microliter between subjects given G-CSF versus G-CSF + GM-CSF only on days 4 and +1 after cytokine administration. Numbers of circulating $CD34^+$ cells started diminishing by 48 hours after the fourth dose of the cytokines.

Few clonogenic cells were present in the PB of these subjects before cytokine administration (7.6 ± 6.3 CFU-GM, 22.2 ± 17.2 BFU-E, and 0.8 ± 0.8 CFU-GEMM per 10^5 low-density mononuclear cells, n = 18). There were no differences among the treatment groups before cytokine administration. After the administration of the first dose of either G-CSF, GM-CSF, or G-CSF + GM-CSF, there was a progressive, significant increase in the number of colony-forming cells in the peripheral blood (Fig 3, A and B). By 48 hours after the first injection of cytokine, the increases in PB CFU-GM colonies were significantly greater after G-CSF or G-CSF + GM-CSF administration than after GM-CSF alone (Fig 3A). Differences between the cytokine regimens with respect to BFU-E and CFU-GEMM were less marked.

One leukapheresis was performed in all subjects 24 hours after the fourth injection of cytokine(s) and the procedure was successfully completed in 15 (two subjects had insuffi-

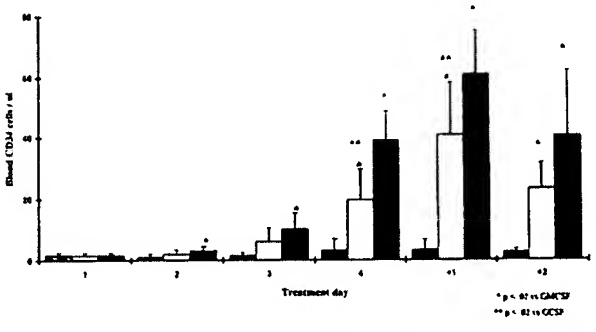


Fig 2. Effect of cytokine administration on the number of $CD34^+$ cells per microliter. GM-CSF (■, n = 5), G-CSF + GM-CSF (□, n = 5), or G-CSF (■, n = 8) were administered after blood was drawn on days 1, 2, 3, and 4.

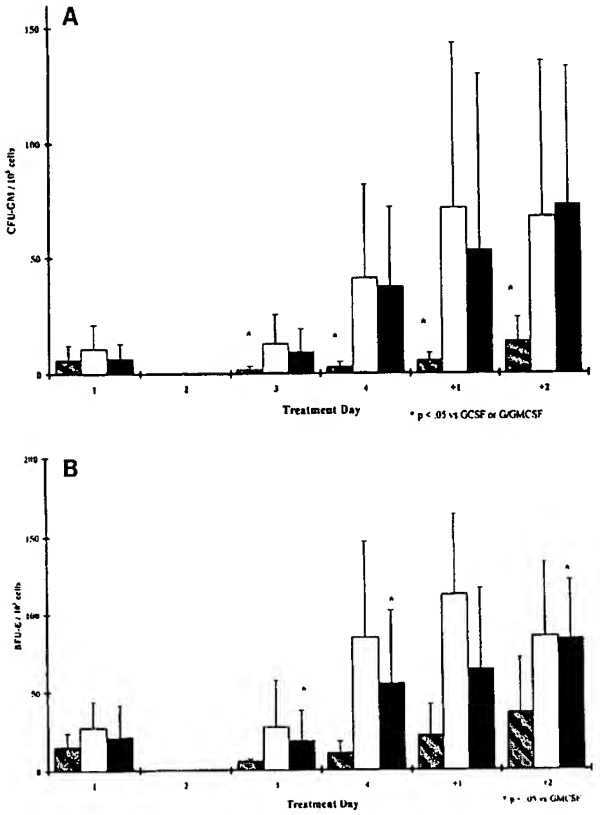


Fig 3. Effect of cytokine administration on the number of clonogenic cells in peripheral blood. (A) CFU-GM; (B) BFU-E. GM-CSF (▩, n = 5), G-CSF + GM-CSF (□, n = 5), or G-CSF (■, n = 8) were administered after blood was drawn on days 1, 2, 3, and 4.

cient venous access for a complete procedure and there was an instrument malfunction on one). Subjects in all three groups had similar yields of leukocytes (G-CSF = 1.9 ± 0.6 × 10^{10}, n = 7; GM-CSF = 1.4 ± 0.2 × 10^{10}, n = 4; G-CSF + GM-CSF = 2.0 ± 0.8 × 10^{10}, n = 4) and mononuclear cells (G-CSF = 1.7 ± 0.5 × 10^{10}; GM-CSF = 1.2 ± 0.1 × 10^{10}; G-CSF + GM-CSF = 1.8 ± 0.6 × 10^{10}). In contrast,

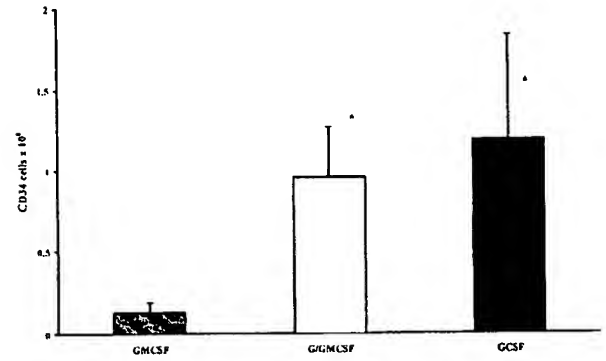


Fig 4. Total CD34⁺ cells collected in the leukapheresis products from patients treated with either GM-CSF (■, n = 4), G-CSF + GM-CSF (□, n = 4), or G-CSF (■, n = 7). P < .05 v GM-CSF.

Table 1. CD34⁺ Cell Subsets in Leukapheresis Harvests From Normal Donors Treated With G-CSF and GM-CSF

Subsets	Treatment		
	G-CSF (n = 4) %	GM-CSF (n = 3) %	G-CSF + GM-CSF (n = 3) %
CD34⁺	1.19 ± 0.33	0.24 ± 0.22*	0.34 ± 0.18
CD34⁺/CD38⁻	0.81 ± 0.22	4.42 ± 3.40	4.73 ± 2.72*
CD34⁺/HLADR⁻	20.7 ± 6.9	20.3 ± 2.9	24.0 ± 9.3
CD34⁺/HLADR⁺/CD38	0.37 ± 0.19	1.10 ± 0.22*	1.86 ± 0.34*

* $P < .05$ v G-CSF.

the overall yield of CD34⁺ mononuclear cells in the leukapheresis product from subjects treated with G-CSF (119 ± 65 × 10^6 cells, n = 7) or with the combination of G-CSF + GM-CSF (101 ± 35 × 10^6 cells, n = 4) was significantly greater than that from subjects treated with GM-CSF (12.6 ± 6.1 × 10^6 CD34⁺ cells, n = 4, $P < .01$ for both comparisons) (Fig 4).

Subset analysis of CD34⁺ cells was performed in 10 of the leukapheresis products (4 mobilized with G-CSF, 3 with GM-CSF, and 3 with G-CSF + GM-CSF). The apheresis products from subjects treated with GM-CSF or G-CSF + GM-CSF contained a significantly higher percentage of CD34⁺ cells that coexpressed HLA-DR but did not coexpress CD38 (CD34⁺/HLA-DR⁺/CD38⁻) compared with those administered G-CSF (Table 1). When converted to absolute numbers, the combination of G-CSF + GM-CSF generated the highest amount of CD34⁺/HLA-DR⁺/CD38⁻ cells in the leukapheresis products (1.41 ± 0.08 × 10^6) versus 0.36 ± 0.21 × 10^6 with G-CSF alone and 0.12 ± 0.05 × 10^6 with GM-CSF ($P < .05$ in all comparisons). There was no significant difference between subjects treated with G-CSF versus GM-CSF with respect to the percentage of CD34⁺/HLA-DR⁻/CD38⁻ cells.

Corresponding to the total number of CD34⁺ cells collected, the number of CFU-GM per leukapheresis was also higher in the G-CSF–mobilized products (Table 2). Interestingly, in subjects treated with G-CSF, the plating efficiencies for CFU-GM (based on the content of CD34⁺ cells) were significantly lower than the plating efficiencies of clonogenic cells collected from subjects treated with GM-CSF (Table 3). A similar, but statistically insignificant, trend was noted for CFU-GEMM ($P = .08$ v GM-CSF). The plating efficiency of BFU-E in subjects treated with G-CSF + GM-CSF was significantly lower than that in subjects treated with either G-CSF or GM-CSF alone.

Table 2. Colony-Forming Potential of Cells Collected by Leukapheresis

Total Colonies Collected × 10^6	G-CSF (n = 7)	GM-CSF (n = 4)	G-CSF + GM-CSF (n = 4)
CFU-GM	11.8 ± 9.4	3.2 ± 2.3*	8.9 ± 2.1
BFU-E	30.3 ± 15.3†	16.2 ± 17.1	13.1 ± 5.0
CFU-GEMM	1.5 ± 0.7	0.9 ± 1.1	0.8 ± 0.4
(CD34⁺ × 10^6)	119 ± 65	12.6 ± 6.1*	101 ± 35

* $P < .05$ v G-CSF and G-CSF + GM-CSF.
† $P < .05$ v G-CSF + GM-CSF.

Table 3. Colony Plating Efficiency Based on Content of CD34⁺ Cells in Leukapheresis Product

Colony Efficiency (% CD34⁺ cells)	G-CSF (n = 7)	GM-CSF (n = 4)	G-CSF + GM-CSF (n = 4)
CFU-GM	11 ± 8	24 ± 11*	10 ± 1
BFU-E	28 ± 12	59 ± 47†	14 ± 3
CFU-GEMM	1.8 ± 0.8	5 ± 5‡	1.8 ± 1.8

* $P < .05$ *v* G-CSF and G-CSF + GM-CSF.
† $P < .05$ *v* G-CSF + GM-CSF.
‡ $P < .05$ *v* G-CSF (single-tail *t*-test).

Before the enrichment of CD34⁺ cells, the percentages of CD34⁺ cells in the G-CSF, GM-CSF, and G-CSF + GM-CSF mobilized products were 1.2% ± 0.4%, 0.2% ± 0.1%, and 0.6% ± 0.3%, respectively. The purity after enrichment was 81% ± 11% for G-CSF (n = 6), 22% ± 7% for GM-CSF (n = 3), and 77% ± 21% for the combination (n = 3), whereas the recovery was 48% ± 12%, 79% ± 32% and 51% ± 15%, respectively. Therefore, the enrichment of CD34⁺ cells was 73- ± 31-fold for the group treated with G-CSF, 138- ± 36-fold for GM-CSF, and 156- ± 86-fold for G-CSF + GM-CSF. The percentages of T and B cells as characterized by CD3, CD5, and CD19 before and after enrichment process are shown in Table 4. The total numbers of T and B cells in each leukapheresis product were reduced to approximately 0.1% of the original. Correspondingly, the clonogenic cells were also enriched after the selection process (Table 5).

DISCUSSION

Recent reports have produced encouraging data to support the use of allogeneic hematopoietic transplantation with the supplementary or exclusive use of blood-derived PCs.[21,22] Several potential advantages are associated with the use of blood-derived PCs versus marrow-derived cells. First, this technique is likely to enhance donor acceptance and eliminates the cost and side effects of general anesthesia.[39,44] Second, the use of PCs mobilized into the PB might be associated with accelerated hematopoietic reconstitution, as extensively shown in the autologous setting.[3,15-18,45] However, blood-derived PC preparations contain a massive number of T cells that might aggravate the development of GHVD.

Table 4. Effect of Enrichment on Lymphocyte Subsets in Leukapheresis Harvests From Normal Donors Treated With G-CSF (n = 10)

Cell Marker	Content of Initial Collection		After CD34⁺ Enrichment		
	% of Cells	Total × 10^8	% of Cells	Total × 10^8	% Reduction
CD3	56 ± 9	4.5 ± 2.0	11 ± 13	4.3 ± 5.2	99.9 ± 0.1
CD5	59 ± 12	4.9 ± 2.3	14 ± 17	5.5 ± 6.3	99.9 ± 0.1
CD19	21 ± 13	1.8 ± 1.4	5 ± 6	1.7 ± 2.0	99.9 ± 0.1

Includes subjects for whom complete data was available, including 5 subjects administered G-CSF, 2 subjects administered GM-CSF, and 3 subjects administered G-CSF + GM-CSF. There were no appreciable differences among groups, hence the data are combined in the table.

Table 5. Enrichment of Clonogenic Cells by CD34⁺ Cell Selection (n = 10)

	CFU-GM	BFU-E	CFU-GEMM
Initial CFU/10^5 cells	46 ± 33	120 ± 88	6 ± 5
Enriched	2,782 ± 2,349	5,558 ± 2,818	168 ± 93
Fold enrichment	68 ± 56	78 ± 60	39 ± 48
Recovery	34 ± 36	32 ± 17*	16 ± 15

Includes subjects for whom complete data were available, including 5 subjects administered G-CSF, 2 subjects administered GM-CSF, and 3 subjects administered G-CSF + GM-CSF. There were no appreciable differences among groups, hence the data are combined in the table.
* $P < .05$ *v* CFU-GEMM.

The present report has demonstrated the feasibility of using PBPC mobilized by G-CSF or G-CSF + GM-CSF for this purpose. We also found that the CD34⁺ PC harvested by apheresis can be significantly enriched and more than 99% of the T cells were eliminated from the enriched product by positive selection of the CD34⁺ cells.

We have shown that, with the current schedule and analysis times, CD34⁺ cells and CFU-GM mobilization are greater with G-CSF than GM-CSF as single agents. The possibility cannot be excluded that if the peak mobilization of CD34⁺ cells or CFU-GM numbers is not yet reached during the time of analysis. Such variables are currently being explored. However, a significantly higher percentage of CD34⁺/HLA-DR⁺/CD38⁻ was found among the CD34⁺ cells mobilized by GM-CSF compared with those by G-CSF alone, suggesting a higher proportion of early progenitors in GM-CSF–mobilized cells. Thus, subjects treated with the combination of G-CSF + GM-CSF showed an equivalent mobilization of CD34⁺ cells and CFU-GM numbers as with G-CSF alone, and also demonstrated a significantly greater mobilization of cells with CD34⁺/CD38⁻ or CD34⁺/HLA-DR⁺/CD38⁻ phenotype. Moreover, the plating efficiency (percent of CD34⁺ cells giving rise to colonies) of CFU-GM as well as BFU-E was higher in cells stimulated by GM-CSF than those by G-CSF. Whether this finding is associated with more rapid engraftment is not known. Nevertheless, this observation shows that CD34⁺ cells are heterogenous, especially when mobilized by different growth factors. The total number of CD34⁺ cells by itself might not correlate with clonogenic assays or with clinical reconstitution. More sophisticated assays analyzing the CD34⁺ subsets are necessary and are currently underway in our laboratory.

Based on experience reported in the literature,[18,19,46] we have adopted the present G-CSF mobilization schedule. However, as the CD34⁺ cells and clonogenic progenitors continued to increase 24 hours after the fourth and final dose of G-CSF or GM-CSF in this study, 1 or more additional days of cytokine administration might eventually give rise to a higher concentration of PC. Weaver et al[19] reported that 16 μg/kg of G-CSF was well tolerated and might result in a better yield of CD34⁺ cells. It is possible that the addition of GM-CSF might stimulate more pluripotent progenitors as discussed above. A combination of these approaches, ie, change in schedule, increase in cytokine dosage, and combinations of cytokines, might improve on the current regimen

such that only one leukapheresis procedure is required.[47,48] Experiments are concurrently underway to address these issues.

The minimal dose of blood-derived hematopoietic PC required for successful engraftment is a critical issue that has yet to be defined. Because the concentration of PCs in PB from normal individuals in steady state is 1/10th to 1/100th of that of bone marrow, "mobilization" procedures using hematopoietic growth factors, alone or in combination with chemotherapy, have been developed for patients with malignancies.[7-14,46,49] For obvious reasons, only the use of growth factors is acceptable for normal donors. It has been suggested that doses of at least $>4 \times 10^8$ mononuclear cells/kg, $>2 \times 10^5$ CFU-GM/kg, and between 2 and 5×10^6 $CD34^+$ cells/kg are required for autologous PC transplantation.[18,50] For allogeneic transplantation the minimum number of blood-derived PC have not been established. Guidelines for donor cell doses when performing allogeneic BMT indicate that a minimum of 1×10^8 mononuclear cells/kg, 5×10^4 CFU-GM/kg, or 2×10^6 $CD34^+$ cells/kg is necessary.[51] With this presumption, our present data suggest that a sufficient number of $CD34^+$ cells for engraftment would have been collected in two of the seven subjects from a single leukapheresis, and in all seven from two leukapheresis procedures. As recovery of $CD34^+$ from the leukapheresis after separation ranged from 25% to nearly 100%, a median of three with a range from two to four leukapheresis procedures might be required using the present mobilization regimen. Weaver et al[19] have recommended even higher doses of $CD34^+$ cells to ensure engraftment. These findings highlight the need to further investigate more effective doses and schedules of mobilizing agents and optimal timing of the apheresis procedures.

Other than the presently described procedure, several methods have been developed to separate and enrich the $CD34^+$ population on a clinical scale. Most of them are based on incubation with MoAbs against CD34 and subsequent binding of the labeled cells using the biotin-avidin system,[52] or cell selection flasks coated with anti-CD34 antibody.[53] Counterflow centrifugal elutriation has also been used to concentrate $CD34^+$ cells and CFU-GM progenitors on a large scale.[54] According to our own preliminary evidence, the use of paramagnetic microspheres has thus far produced one of the purest products (99.2%; unpublished results, June 1994). However, we are not yet aware of any data on comparisons of these various methods.

The incidence and severity of GVHD in the setting of allogeneic transplantation is dependent on the number of host T cells administered to the recipient.[55] The positive selection technique used in this study removed up to 3 logs (99.9%) of the T cells originally present in the apheresis product and resulted in approximately 5×10^6 residual T cells in the enriched product. This would result in an average dose of 0.7×10^5 T cells/kg to a 70-kg allogeneic transplant recipient and is well below the 1 to 5×10^5 T cells/kg range reported to reduce GVHD.[55,56] The role of T-cell depletion in allogeneic transplant is still controversial. The use of a technique which separates $CD34^+$ cells from the remaining cells harvested from the PB should facilitate the investigation of the role of specific T-cell subsets in mediating engraftment while minimizing GVHD.

ACKNOWLEDGMENT

The authors thank Mary Cobb, Barbara Vickers, and Susan Shinagawa for their excellent administrative assistance; Dr David Ward and Odette Ada, RN, for performing the leukapheresis procedures; and the staff of the UCSD Clinical Research Center for the daily care of the experimental subjects.

REFERENCES

1. Körbling M, Dorken B, Ho AD, Pezzutto A, Hunstein W, Fliedner TM: Autologous transplantation of blood-derived stem cells after myeloablative therapy in a patient with Burkitt's lymphoma. Blood 67:529, 1986

2. Kessinger A, Armitage JO, Landmark JD: Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. Blood 71:723, 1988

3. Juttner CA, To HO, Ho JQ, Bardy PG, Dyson PG, Haylock DN, Kimber RJ: Early lymphohematopoietic recovery after autografting using peripheral blood stem cells in acute non-lymphoblastic leukemia. Transplant Proc 20:40, 1988

4. Richman CM, Weiner RS, Yankee RA: Increase in circulating stem cells following chemotherapy in man. Blood 47:1031, 1976

5. To LB, Haylock DN, Kimber RJ, Juttner CA: High levels of circulating haematopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. Br J Haematol 58:399, 1984

6. To LB, Shepperd KM, Haylock DN, Dyson PG, Charles P, Thorp DL, Dale BM, Dart GW, Roberts MM, Sage RE, Juttner CA: Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. Exp Hematol 18:422, 1990

7. Siena S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni AM: Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide treated patients: Enhancement by intravenous recombinant human granulocyte-macrophage colony stimulating factor. Blood 74:1905, 1989

8. Giani AM, Siena S, Bregni M, Tarella C, Stern AC, Pileri A, Bonadonna G: Granulocyte-macrophage colony stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. Lancet 2:580, 1989

9. Ho AD, Haas R, Körbling M, Dietz M, Hunstein W: Utilization of recombinant human GM-CSF to enhance peripheral progenitor cell yield for autologous transplantation. Bone Marrow Transplant 7:13, 1991 (suppl 1)

10. Elias AD, Ayash L, Anderson KC, Hunt M, Wheeler C, Schwartz G, Tepler I, Mazanet R, Lynch C, Pap S, Peluez J, Reich E, Critchlow J, Demetri G, Bibbo J, Schnipper L, Griffin JD, Frei E III, Antman K: Mobilization of peripheral blood progenitor cells by chemotherapy and granulocyte-macrophage colony stimulating factor for hematologic support after high-dose intensification for breast cancer. Blood 79:3036, 1992

11. Haas R, Ho AD, Bredthauer U, Cayeux S, Egerer G, Knauf W, Hunstein W: Successful autologous transplantation of blood stem cells mobilized with recombinant human granulocyte-macrophage colony-stimulating factor. Exp Hematol 18:94, 1990

12. Socinski MA, Elias A, Schnipper L, Cannistra SA, Antman KH, Griffin JD: Granulocyte-macrophage colony stimulating factor expands the circulating haemopoietic progenitor cell compartment in man. Lancet 1:1194, 1988

13. Brugger W, Bross K, Frisch J, Dern P, Weber B, Mertelsmann R, Kanz L: Mobilization of peripheral blood progenitor cells by sequential administration of interleukin-3 and granulocyte-macro-

phage colony-stimulating factor following polychemotherapy with etoposide, ifosfamide, and cisplatin. Blood 79:1193, 1992

14. Henon PR, Becker M: Cytokine enhancement of peripheral blood stem cells. Stem Cells 11:65, 1993 (suppl 2)

15. Advani R, Chao NJ, Horning SJ, Blume IG, Ahn DK, Lamborn KR, Fleming NC, Bonnem EM, Greenberg PL: Granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjunct to autologous hemopoietic stem cell transplantation for lymphoma. Ann Intern Med 116:183, 1992

16. Lasky LC, Hurd DD, Smith JA, Haake R: Peripheral blood stem cell collection and use in Hodgkin's disease. Comparison with marrow in autologous transplantation. Transfusion 29:323, 1989

17. To LB, Roberts MM, Haylock DN, Dyson PG, Branford AL, Thorp D, Ho JQ, Dart GW, Horvath N, Davy ML, Olweny CL, Abdi E, Juttner CA: Comparison of hematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. Bone Marrow Transplant 9:277, 1992

18. Bensinger W, Singer J, Appelbaum F, Lilleby K, Longin K, Rowley S, Clarke E, Clift R, Hansen J, Shields T, Storb R, Weaver C, Welden P, Buckner CD: Autologous transplantation with peripheral blood mononuclear cells collected after administration of recombinant granulocyte stimulating factor. Blood 81:3158, 1993

19. Weaver CH, Buckner CD, Longin K, Appelbaum FR, Rowley S, Lilleby K, Miser J, Störb R, Hansen JA, Bensinger W: Syngeneic transplantation with peripheral blood mononuclear cells collected after the administration of recombinant human granulocyte colony-stimulating factor. Blood 82:1981, 1993

20. Kessinger A, Smith DM, Strandjord SE, Landmark JF, Dooley DC, Law P, Coccia PF, Warkentin PI, Weisenburger DD, Armitage JO: Allogeneic transplantation of blood-derived T cell depleted hematopoietic stem cells after myeloablative treatment in a patient with acute lymphoblastic leukemia. Bone Marrow Transplant 4:643, 1989

21. Russell NH, Hunter A, Rogers S, Hanley J, Anderson D: Peripheral blood stem cells as an alternative to marrow for allogeneic transplantation (letter). Lancet 341:1482, 1993

22. Neumunaitis J, Rosenfeld C, Collins R, Pineiro L, Ohr S, Pallansch P, Shadduck R, Stone M, Fay J: Pilot trial of allogeneic transplant combining peripheral blood and bone marrow in patients with refractory hematologic malignancy. Blood 82:417a, 1993 (abstr, suppl 1)

23. Hershko C, Gale RP, Ho WG, Cline MJ: Cure of aplastic anaemia in paroxysmal nocturnal haemoglobinukria by marrow transfusion from identical twin: Failure of peripheral-leukocyte transfusion to correct marrow aplasia. Lancet 1:945, 1979

24. Abrams RA, Glaubiger D, Appelbaum FR, Deisseroth AB: Result of attempted hematopoietic reconstitution using isologous, peripheral blood mononuclear cells: A case report. Blood 56:516, 1980

25. Ho AD, Glück S, Germond C, Sinoff C, Dietz G, Maruyama M, Corringham RET: Optimal timing for collections of blood progenitor cells following induction chemotherapy and granulocyte-macrophage colony-stimulating factor for autologous transplantation in advanced breast cancer. Leukemia 7:1738, 1993

26. Siena S, Bregni M, Brando B, Belli N, Ravagnani F, Gandola L, Stern AC, Lansdorp PM, Bonadonna G, Gianni AM: Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. Blood 77:400, 1991

27. Dreger P, Marquardt P, Haferlach T, Jacobs S, Mulverstedt T, Eckstein V, Suttorp M, Loffler H, Muller-Ruchholtz W, Schmitz N: Effective mobilization of peripheral blood progenitor cells with "Dexa-BEAM" and G-CSF: Timing of harvesting and composition of the leukapheresis product. Br J Cancer 68:950, 1993

28. Matsunaga T, Sakamaki S, Kohgo Y, Ohi S, Hirayama Y, Nitsu Y: Recombinant human granulocyte colony stimulating factor can mobilize sufficient amounts of peripheral blood stem cells in healthy volunteers for allogeneic transplantation. Bone Marrow Transplant 11:103, 1993

29. Widman FK (ed): Standards for Blood Banks and Transfusion Services (ed 15). Bethesda, MD, American Association of Blood Banks, 1993

30. Huang S, Terstappen LWMM: Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. Nature 360:745, 1992

31. Terstappen LWMM, Mickails R, Dost R, Loken MR: Increased light scattering resolution facilitates multidimensional flow cytometric analysis. Cytometry 11:506, 1990

32. Terstapen LWMM, Huang S, Picker LJ: Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. Blood 79:666, 1992

33. Bender JG, Unverzagt KL, Walker DE, Lee W, Van Epps DE, Smith DH, Stewart CC, To LB: Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. Blood 77:2591, 1991

34. Iscove NN: Colony formation by normal and leukemic marrow cells in culture: Effect of conditioned medium from human leukocytes. Blood 37:1, 1971

35. Civin CI, Banquerigo ML, Strauss LC, Loken MR: Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of My-10-positive progenitor cells in normal human bone marrow. Exp Hematol 15:10, 1987

36. Civin CI: Human monomyeloid cell membrane antigens. Exp Hematol 18:461, 1990

37. Strauss LC, Trischmann TM, Rowley SD, Wiley JM, Civin CI: Selection of normal human hematopoietic stem cells for bone marrow transplantation using immunomagnetic microspheres and CD34 antibody. Am J Pediatr Hematol Oncol 2:217, 1991

38. Ishizawa L, Hangoc G, Van de Ven C, Cairo M, Burgess J, Mansour V, Gee A, Hardwick A, Traycoff C, Srour E, Hoffman R, Law P: Immunomagnetic separation of CD34+ cells from human bone marrow, cord blood, and mobilized peripheral blood. J Hematother 2:333, 1993

39. Lowry PA, Tabbara IA: Peripheral hematopoietic stem cell transplantation: Current concepts. Exp Hematol 20:937, 1992

40. Juttner CA, To LB, Haylock DN, Branford A, Kimber RJ: Circulating autologous stem cells collected in very early remission from acute nonlymphoblastic leukemia produce prompt but incomplete haemopoietic reconstitution after high dose melphalan or supralethal chemoradiotherapy. Br J Haematol 61:739, 1985

41. Kessinger A, Armitage JO: The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies. Blood 77:211, 1991

42. Eaves CJ: Peripheral blood stem cells reach new heights. Blood 82:1957, 1993

43. Buckner CD, Clift RA, Sanders JE, Stewart P, Bensinger WI, Doney KC, Sullivan KM, Witherspoon RP, Deeg HJ, Appelbaum FR, Störb R, Thomas ED: Marrow harvesting from normal donors. Blood 64:630, 1984

44. Bortin MM, Buckner CD: Major complications of marrow harvesting for transplantation. Exp Hematol 11:916, 1983

45. Sheridan WP, Begley CG, Juttner CA, Szer J, To LB, Maher D, McGrath KM, Morstyn G, Fox RM: Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. Lancet 339:640, 1992

46. McCarthy DM, Goldman JM: Transfusion of circulating stem cells. CRC Crit Rev Clin Lab Sci 20:1, 1984

47. Peters WP, Rosner G, Ross M, Vredenburgh J, Meisenberg

B, Gilbert C, Kurtzberg J: Comparative effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) on priming peripheral blood progenitor cells for use with autologous bone marrow after high-dose chemotherapy. Blood 81:1709, 1993

48. Mason J, Ho A, Mullen M, Young D, Shea T: High-dose carboplatin with peripheral blood stem cells and sequential interleukin-3 and GM-CSF. Blood 82:365a, 1993 (abstr, suppl 1)

49. McNiece IK, Briddell RA, Hartley CA, Smith KA, Andrews RG: Stem cell factor enhances in vivo effects of granulocyte colony stimulating factor for stimulating mobilization of peripheral blood progenitor cells. Stem Cells 11:36, 1993 (suppl 2)

50. Bender JG, To LB, Williams S, Schwartzberg LS: Defining a therapeutic dose of peripheral blood stem cells. J Hematother 1:329, 1992

51. Storb R, Prentice RL, Thomas ED, Appelbaum FR, Deeg HJ, Doney K, Fefer A, Goodell BW, Mickelson E, Stewart P, Sullivan KM, Witherspoon RP: Factors associated with graft rejection after HLA-identical marrow transplantation for aplastic anaemia. Br J Haematol 55:573, 1983

52. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID: Antigen CD34$^+$ marrow cells engraft lethally irradiated baboons. J Clin Invest 81:951, 1988

53. Lebkowzki J, Schain LR, Okrongly D, Levinsky R, Harvey MJ, Okarma TB: Rapid isolation of human CD34 hematopoietic stem cells—Purging of human tumor cells. Transplantation 53:1011, 1992

54. Hillyer CD, Lackey DA, Hart KK, Stempora LL, Bray RA, Bender JG, Donnenberg AD: CD34$^+$ progenitors and colony-forming units-granulocytic macrophage are recruited during large-volume leukapheresis and concentrated by counterflow centrifugal elutriation. Transfusion 33:316, 1993

55. Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ: Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. Blood 68:770, 1986

56. Atkinson K, Farrelly H, Cooley M, O'Flaherty E, Downs K, Biggs J: Human marrow T cell dose correlates with severity of subsequent acute graft-versus-host disease. Bone Marrow Transplant 2:51, 1987

Exhibit 3

Cycling Status of CD34+ Cells Mobilized Into Peripheral Blood of Healthy Donors by Recombinant Human Granulocyte Colony-Stimulating Factor

By Roberto M. Lemoli, Agostino Tafuri, Alessandra Fortuna, Maria Teresa Petrucci, Maria Rosaria Ricciardi, Lucia Catani, Damiano Rondelli, Miriam Fogli, Giuliana Leopardi, Cristina Ariola, and Sante Tura

In this study, we assessed the functional and kinetic characteristics of highly purified hematopoietic CD34+ cells from the apheresis products of 16 normal donors undergoing glycosylated granulocyte colony-stimulating factor (G-CSF) treatment for peripheral blood stem cells (PBSC) mobilization and transplantation in allogeneic recipients. Mobilized CD34+ cells were evaluated for their colony-forming capacity and trilineage proliferative response to selected recombinant human (rh) CSF in vitro and the content of very primitive long-term culture initiating cells (LTC-IC). In addition, the cycling status of circulating CD34+ cells, including committed clonogenic progenitor cells and the more immature LTC-IC, was determined by the cytosine arabinoside (Ara-C) suicide test and the acridine orange flow cytometric technique. By comparison, bone marrow (BM) CD34+ cells from the same individuals were studied under steady-state conditions and during G-CSF administration. Clonogenic assays in methylcellulose showed the same frequency of colony-forming unit cells (CFU-C) when PB-primed CD34+ cells and BM cells were stimulated with phytohemagglutinin-lymphocyte-conditioned medium (PHA-LCM). However, mobilized CD34+ cells were significantly more responsive than their steady-state BM counterparts to interleukin-3 (IL-3) and stem cell factor (SCF) combined with G-CSF or IL-3 in presence of erythropoietin (Epo). In cultures added with SCF, IL-3, and Epo, we found a mean increase of 1.5- ± 1-fold (standard error of the mean [SEM]) of PB CFU-granulocyte-macrophage and erythroid progenitors (burst-forming units-erythroid) as compared with BM CD34+ cells ($P < .05$). Conversely, circulating and BM megakaryocyte precursors (CFU-megakaryocyte) showed the same clonogenic efficiency in response to IL-3, granulocyte-macrophage-CSF and IL-3, IL-6, and Epo. After 5 weeks of liquid culture supported by the engineered murine stromal cell line M2-10B4 to produce G-CSF and IL-3, we reported 48.2 ± 35 (SEM) and 62.5 ± 54 (SEM) LTC-IC per 10^4 CD34+ cells in PB and steady-state BM, respectively (P = not significant). The Ara-C suicide assay showed that 4% ± 5% (standard deviation [SD]) of committed precursors and 1% ± 3% (SEM) of LTC-IC in PB are in S-phase as compared with 25.5% ± 12% (SD) and 21% ± 8% (SEM) of baseline BM, respectively ($P < .001$). However, longer incubation with Ara-C (16 to 18 hours), in the presence of SCF, IL-3 and G-CSF, or IL-6, showed that more than 60% of LTC-IC are actually cycling, with no difference being found with BM cells. Furthermore, studies of cell-cycle distribution on PB and BM CD34+ cells confirmed the low number of circulating progenitor cells in S- and G_2M-phase, whereas simultaneous DNA/RNA analysis showed that the majority of PB CD34+ cells are not quiescent (ie, in G_0-phase), being in G_1-phase with a significant difference with baseline and G-CSF-treated BM (80% ± 5% [SEM] *v* 61.9% ± 6% [SEM] and 48% ± 4% [SEM], respectively; $P < .05$). Moreover, G-CSF administration prevented apoptosis in a small but significant proportion of mobilized CD34+ cells. Thus, our results indicate that mobilized and BM CD34+ cells can be considered equivalent for the frequency of both committed and more immature hematopoietic progenitor cells, although they show different kinetic and functional profiles. In contrast with previous reports, we found that PB CD34+ cells, including very primitive LTC-IC, are cycling and ready to progress into S-phase under CSF stimulation. This finding should be taken into account for a better understanding of PBSC transplantation.

SUCCESSFUL TRANSPLANTATION of allogeneic recombinant human granulocyte colony-stimulating factor (rhG-CSF)-mobilized peripheral blood stem cells (PBSC) has recently been reported by several groups.[1-3] As with autografting, the most striking finding of PBSC transplantation was the faster reconstitution of hematopoiesis after myeloablative conditioning regimen as compared with bone marrow (BM)-derived stem cells.[1] The results of these clinical trials raise the question as to whether circulating progenitor cells may differ from their BM counterparts with respect to cell-cycle characteristics, immunophenotype, frequency of both committed and very primitive precursors, and their proliferative response upon stimulation with cytokines. Few reports so far have addressed these issues in the setting of allogeneic transplantation.[4-7] One early report[4] showed a high expression of myeloid antigens on PB CD34+ cells at the expense of B-lineage-associated antigens (ie, CD10, CD19, and CD20) coupled with a high colony-forming capacity of G-CSF-stimulated apheresis products. More recent investigations[5,7] have shown that only a small minority of murine and human circulating progenitor cells (either in steady-state or after priming with G-CSF) is in the S-phase of the cell cycle as compared with BM cells. Thus, it was concluded that progenitors mobilized into PB after cytokine treatment are noncycling, whereas hematopoietic precursors in BM are actively proliferating. However, these seminal studies did not analyze either the cell-cycle distribution of hematopoietic stem cells or the activity of G-CSF on CD34+ cells programmed cell death. In addition, it is still unclear whether the most immature subsets within mobilized PBSC show the same functional and kinetic properties as BM cells. It is, in fact, very important to quantify and characterize the

From the Institute of Hematology "L. & A. Seràgnoli" and the Institute of Radiotherapy, University of Bologna, Bologna, Italy; and the Institute of Hematology, Department of Human Biopathology, University "La Sapienza" of Rome, Rome, Italy.

Submitted May 17, 1996; accepted September 30, 1996.

R.M.L. and A.T. contributed equally to this work.

Supported by MURST 40%, MURST 60%, Associazione Italiana per la Ricerca Contro il Cancro (AIRC; Milan, Italy), and Fondazione Cenci-Bolognetti. M.R.R. is supported by AIRC.

Address reprint requests to Roberto M. Lemoli, MD, Institute of Hematology "L. & A. Seràgnoli", Via Massarenti, 9, 40138 Bologna, Italy.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/97/8904-0017$3.00/0

CV1

cellular populations that are believed to ensure permanent engraftment after PBSC allografting, along with the more committed progenitor cells responsible for short-term BM reconstitution. In this regard, the long-term culture initiating cells (LTC-IC) are the most reliable approximation to true stem cells in humans.[8]

To further elucidate the functional and kinetic characteristics of G-CSF–mobilized hematopoietic progenitor cells, we have analyzed highly purified CD34$^+$ cells from the apheresis products of 16 normal subjects undergoing PBSC collection for allogeneic transplantation. The results were then compared with those obtained on CD34$^+$ cells enriched from the BM of the same donors under steady-state conditions and during G-CSF administration on the same day of PBSC harvest. The results presented here indicate that PB CD34$^+$ cells show the same frequency of colony-forming units-cells (CFU-C) and LTC-IC as BM samples. The percentage of both committed and very primitive hematopoietic precursors in the S/G_2M-phase is significantly lower than that observed in the BM at baseline or after G-CSF administration. However, the vast majority of PB CD34$^+$ cells, including the more immature LTC-IC, are cycling and myeloid precursors show increased responsiveness to additional cytokines in vitro. Moreover, G-CSF treatment significantly protects PB CD34$^+$ cells from apoptosis.

MATERIALS AND METHODS

PBSC donors and mobilization protocol. PB and BM samples were obtained from 16 healthy adults (6 men and 10 women, 18 to 55 years old). The donors received glycosylated rhG-CSF (Lenograstim; Rhone-Poulenc Rorer, Milan, Italy) administered subcutaneously at 12 μg/kg/d for 5 to 6 days for mobilization and collection of PBSC before allogeneic transplantation. All donors were related and had HLA-full match with the recipient. Inclusion criteria were good general health (Eastern Cooperative Oncology Group performance status = 0), normal full blood examination, normal coagulation profile, and normal renal and liver functions. Normal subjects were excluded from the study in case of pregnancy or lactation, positive serology for hepatitis C and B virus, human immunodeficiency virus positivity, or any major organ or system dysfunction. Hematologic indices and the number of CD34$^+$ cells in PB were recorded at baseline and daily until the completion of PBSC collection. Leukapheresis were performed on days 5 and 6 using either a Fenwal CS3000 continuous flow blood cell separator (Baxter Healthcare Corp, Deerfield, IL), as previously reported,[9] or a Cobe Spectra separator (Cobe BCT, Inc, Lakewood, CO). Apheresis products were then cryopreserved in liquid nitrogen until transplantation.[9] No donor required central line placement to undergo apheresis. All normal subjects successfully completed mobilization and collection of PBSC. Hematopoietic CD34$^+$ cell purification and subsequent studies were always performed using day-5 collections at the peak time of PB CD34$^+$ cells. BM specimens were obtained before G-CSF mobilization, in steady-state conditions, and (in selected cases) during G-CSF treatment on the same day as PBSC harvest. To minimize PB cell contamination, no more than 2 to 3 mL of BM was collected from each aspiration. The protocol was approved by the ethical commitee of the University Hospital and each normal donor gave written informed consent.

Cell preparation and CD34$^+$ cell purification. Mononuclear cells were obtained by gradient centrifugation (Lymphoprep; 1.077 g/mL; Nycomed Pharma, Oslo, Norway). Light-density cells were washed twice in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and CD34$^+$ cells were highly purified by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.[10] To assess the percentage of CD34$^+$ elements, aliquots of the CD34$^+$ target cells were restained with an antibody (HPCA-2; IgG1a-fluorescein isothiocyanate [FITC]; Becton Dickinson, San Jose, CA) directed at a different epitope of CD34 antigen than that (QBend10) used with the MiniMacs system. Briefly, CD34$^+$ cells were incubated for 30 minutes in the dark at 4°C with HPCA-2-FITC. Propidium iodide (2 μg/mL) was added for the detection of nonviable cells, which were excluded from analysis. After two washes in PBS/BSA, flow cytometric analysis was performed on a gated population set on scatter properties by using FACScan equipment (Becton Dickinson). A minimum of 10,000 events was collected in list mode on FACScan software.

Colony assays. Normal BM and PB cells were cultured in semisolid medium as previously described.[11] Briefly, 1,000 to 5,000 CD34$^+$ cells were plated in duplicate in culture medium consisting of 1 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 24% fetal calf serum (Sera Lab, Crawley Down, Sussex, UK), 0.8% BSA (Sigma), 10^{-4} mol/L 2-mercaptoethanol (Sigma), 2 U recombinant human erythropoietin (Epo; Dompè Biotec, Milan, Italy), and 0.2 mmol/L bovine hemin. To measure the optimum clonogenic efficiency, 10% (vol/vol) of a selected lot of phytohemagglutinin–lymphocyte-conditioned medium (PHA-LCM) was added. The final concentration of methylcellulose was 1.1%. Granulocyte-macrophage CFU (CFU-GM), erythroid progenitors (burst-forming unit-erythroid [BFU-E]), and mixed colonies (CFU-granulocyte, erythroid, monocyte, megakaryocyte [CFU-GEMM]) were scored after 14 days of incubation at 37°C in a fully humidified 5% CO_2 atmosphere. When indicated, PHA-LCM was replaced by combinations of cytokines including the following recombinant human CSFs: G-CSF (1,000 U/mL), interleukin-3 (IL-3; 50 ng/mL; Genetics Institute, Cambridge, MA), and stem cell factor (SCF; 50 ng/mL; Amgen, Thousand Oaks, CA). All cultures were performed in the presence of 2 U/mL of Epo.

Megakaryocyte progenitor cells (CFU-megakaryocyte [CFU-MK]) derived from highly purified CD34$^+$ cells were grown in plasma clot cultures as reported elsewhere.[11] Recombinant human cytokines were added at the following concentrations: IL-3 at 10 ng/mL, Epo at 2 U/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF; Amgen) at 2 ng/mL, and IL-6 (Genzyme) at 100 ng/mL. After 12 to 14 days of incubation, plasma clots were fixed with methanol-acetone (1:3) for 20 minutes, washed with PBS, and air dried. Fixed dishes were stored at −20°C until immunofluorescence staining was performed. CFU-MK colonies were recorded as strongly positive aggregates after staining with a monoclonal antibody directed to the glycoprotein complex IIb-IIIa (CD41; Dako, Glostrup, Denmark). Binding was assessed by FITC-goat antimouse IgG (Ortho, Milan, Italy).[11]

LTC-IC assay. The number of LTC-IC was determined from the leukapheresis products and the BM, as previously described,[12] with some modifications. Briefly, 10,000 to 20,000 highly purified CD34$^+$ cells/mL of medium were seeded onto preestablished irradiated genetically engineered murine stromal cells.[13] The murine marrow-derived stromal cell line M2-10B4 (kind gift of Dr C. Carlo-Stella, University of Parma, Parma, Italy) was genetically engineered by retroviral-mediated gene transfer to produce G-CSF and IL-3 and was used in all experiments to optimize and standardize the clonogenic output per LTC-IC. The growth medium consisted of 12.5% horse serum (Sera Lab), 12.5% fetal calf serum, and 10^{-6} mol/L hydrocortisone sodium succinate (Sigma) in IMDM supplemented with 1% antibiotics and glutamine. Cultures were incubated at 37°C in a fully humidified 5% CO_2 atmosphere with weekly half-medium

change. After 5 weeks in culture, nonadherent and adherent cells were pooled, washed, and plated together in methylcellulose for the determination of total progenitor cell content (CFU-GM, BFU-E, and CFU-GEMM), and the number of LTC-IC was calculated as earlier reported.[12]

Cytosine arabinoside (Ara-C) suicide assay for S-phase analysis. To evaluate the proportion of hematopoietic progenitors in S-phase, the Ara-C suicide test was performed by incubating 1×10^5 cells/mL with 2×10^{-6} mol/L Ara-C (Aracytin; Upjohn, Kalamazoo, MI) for 1 hour at 37°C, as previously reported.[11] The cycling status of LTC-IC was determined by incubating CD34+ cells with Ara-C for 16 to 18 hours in the presence of 20 ng/mL of SCF, IL-3, and G-CSF[14] in serum-containing medium or in serum-free conditions.[15] In selected experiments, G-CSF was replaced by 1,000 U/mL of IL-6, and the results were found to be superimposable. After two washes, CD34+ cells were plated in semisolid medium (see clonogenic assays) or grown in liquid culture (see LTC-IC assay), and the difference in the number of colonies between treated and untreated cultures, representing the percentage of BM and PB precursors undergoing DNA synthesis, was calculated after 2 and 5 weeks, respectively.

Cell-cycle studies. To evaluate the cell-cycle distribution of CD34+ cells, the acridine orange (AO) flow cytometric technique was used. Cellular DNA and RNA content (percentage of cells in G_0, G_1, S, and G_2M; mean RNA content of cells in each phase of the cell cycle) was measured as previously reported.[15] Discrimination between G_0 and G_1-phase of cell cycle was made on the basis of RNA cellular content. G_0 cells were defined as cells with an RNA content equal or lower than that of G_1 cells and equal to that of control lymphocytes. The RNA content of G_1 cells was expressed as the RNA index (RNA-I G_1), which was determined as the ratio of the mean RNA content of G_1 cells of the samples times 10, divided by the median RNA content of control lymphocytes.[16,17] Moreover, we used the AO assay to evaluate the number of apoptotic cells as a sub-G_1 peak on the DNA frequency histograms, because these cells can be recognized by their diminished stainability with DNA-specific fluorochromes.[15,18] In fact, AO staining allows discrimination between necrotic and apoptotic cells because of decreased stainability of apoptotic elements in DNA green fluorescence coupled with a higher red fluorescence (which is common to chromatin condensation and higher content of single-stranded DNA).[18] Cell debris were excluded from analysis on the basis of their forward light scatter properties. Modified FACScan equipment (Becton Dickinson) was used to measure fluorescence upon excitation at 488 nm. Five thousand cells were measured for each analysis at separate wavelength bands for green/DNA and red/RNA. Samples were analyzed using a Hewlett Packard microcomputer and Becton Dickinson software including Consort 32, Cellfit, and Lysis II.

Statistical analysis. The results are expressed as the mean ± the mean of the standard error (SEM) or the standard deviation (SD), as indicated. Statistical analysis was performed by mean of the nonparametric paired Wilcoxon rank-sum test.

Table 1. Purification of CD34+ Cells From the BM and Leukapheresis Products of Healthy Donors Treated With rhG-CSF

Source of Cells	% Start	% Purity	% Recovery	Enrichment Factor
PB (D5)	0.7 ± 0.4	98.9 ± 0.8	90 ± 7.7	137 ± 46
BM (D0)	2.5 ± 1*	95 ± 6	87.9 ± 15	57 ± 28*
BM (D5)	2.1 ± 1*	96 ± 4	88 ± 12	57 ± 22*

The results are expressed as the mean ± SD of 26 experiments (BM = 10).

* Statistically significant compared with PB samples.

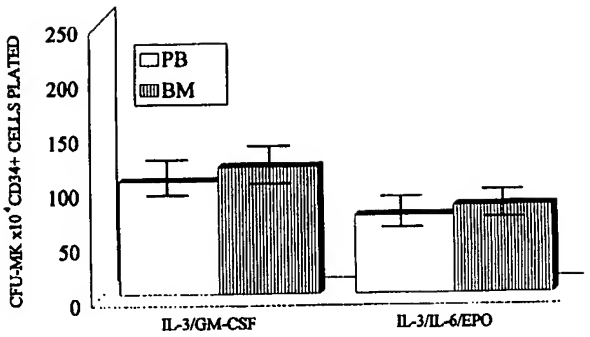


Fig 1. Comparative clonogenic growth of megakaryocyte progenitor cells (CFU-MK) derived from PB and baseline BM CD34+ cells upon incubation with rhCSFs. The results are expressed as the mean ± SEM of the number of CFU-MK colonies per 10^4 CD34+ cells plated.

RESULTS

Collection and enrichment of CD34+ cells. Sixteen normal donors underwent G-CSF treatment and PBSC collection for allogeneic transplantation. The number of CD34+ cells peaked in all but 1 healthy subject at day 5 (data not shown), when PBSC harvests were initiated. Positive selection of CD34+ cells was always performed using PBSC samples recovered at day 5. Ten donors agreed to donate BM cells for purification and analysis of hematopoietic progenitors cells before and during G-CSF administration (D0 and D5, respectively). The percentage of CD34+ cells in BM (D0 and D5) and PB samples was 2.5% ± 1% (SD), 2.1% ± 1% (SD), and 0.7% ± 0.4% (SD) of the mononuclear cell fraction, respectively. After magnetic separation, the percentage of CD34+ in BM was 95% ± 6% (SD) and 96% ± 4% (SD) at D0 and D5, respectively, and in PB was 98.9% ± 0.8% (SD). The mean enrichment factors were 57-fold in BM and 137-fold in PB. The mean overall recovery of CD34+ cells was greater than 87% (Table 1). When we analyzed the coexpression on CD34+ cells of lineage-associated antigens as well as selected activation molecules, we found that greater than 90% of hematopoietic cells in both compartments coexpressed the CD38 and HLA-DR antigens, whereas there was a significant increase in the proportion of PB myeloid cells (ie, CD34+/CD33+ and CD34+/CD13+) at the expense of B-lymphocyte precursors (CD34+/CD19+), as already reported[4] (data not shown).

Frequency and proliferative response of CFU-C to rhCSFs. All of the tested CD34+ cell fractions showed significant colony formation when stimulated with PHA-LCM and Epo. The clonogenic efficiency of CFU-GM and BFU-E was 1.4% ± 0.3% (SEM) and 2.5% ± 0.5% (SEM) for PB-derived samples and 1.4% ± 0.4% (SEM) and 2.2% ± 0.3% (SEM) for BM cells, respectively. Thus, no significant difference was noted in the frequency (number of colonies formed per number of cells plated) of CFU-C in primed PB versus steady-state BM when a conditioned medium was used for colony growth stimulation. We then assessed the trilineage proliferation of PB and baseline BM CD34+ cells in response to selected CSFs (Figs 1 and 2). Comparative experiments on colony formation of megakaryocyte precur-

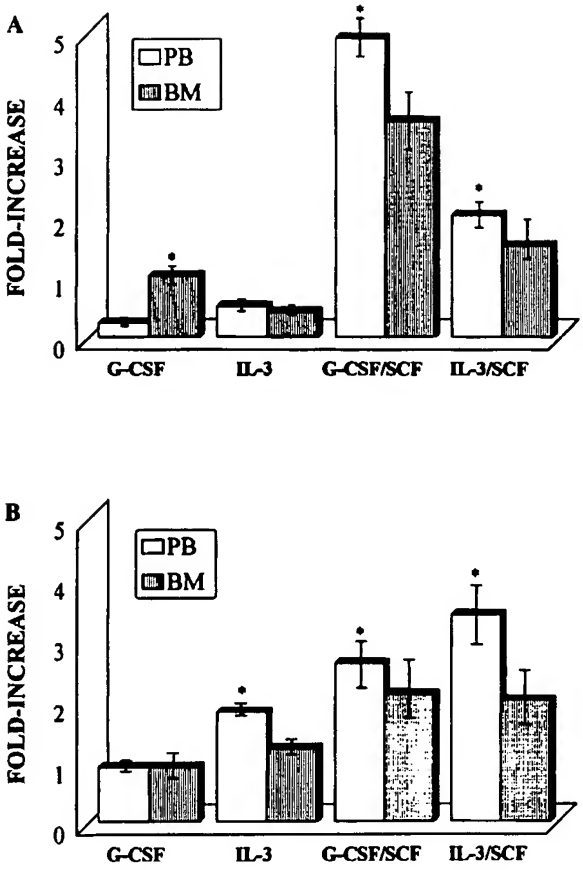


Fig 2. Proliferative response of CFU-GM (A) and BFU-E (B) derived from highly purified PB and steady-state BM CD34⁺ cells to recombinant human growth factors. The results are expressed as the mean ± SEM of the fold increase compared with PHA-LCM–supplemented cultures. The clonogenic efficiency of control (PHA-LCM) dishes was 1.4% ± 0.3% (CFU-GM) and 2.5% ± 0.5% (BFU-E) for PB CD34⁺ cells and 1.4% ± 0.4% (CFU-GM) and 2.2% ± 0.3% (BFU-E) for BM CD34⁺ cells, respectively ($P > .5$). All of the experiments were performed in the presence of 2 U/mL of Epo. *Statistically significant.

sors (CFU-MK) in the presence of IL-3 and GM-CSF or IL-3, IL-6, and Epo did not show any significant difference between the two compartments (Fig 1). Similar data were obtained when the more immature BFU-MK were assayed (data not shown). Conversely, the results presented in Fig 2 show that priming with G-CSF significantly increased the number of PB CFU-GM stimulated both by G-CSF/SCF and IL-3/SCF ($P < .05$) and BFU-E cultured with IL-3 and CSF combinations in the presence of Epo ($P < .05$). The more pronounced effect was seen on IL-3/SCF–responsive CFU-C (1.5- ± 1-fold increase in PB *v* BM CD34⁺ cells; $P < .02$). CD34⁺ cells enriched from steady-state BM generated more CFU-GM colonies than did their circulating counterparts when stimulated with G-CSF alone ($P < .04$). Notably, the addition of SCF to culture media increased additively or synergistically the number and size of CFU-C induced by G-CSF or IL-3 (Fig 2). Taken together, these results suggest a higher responsiveness of mobilized CD34⁺ cells to selected growth factors as compared with steady-state BM cells. However, previous studies from our laboratory showed that pretreatment with G-CSF also modifies the response of BM hematopoietic progenitor cells to subsequent stimulation with additional growth factors.[11] Thus, G-CSF seems to exert its priming effect in a similar way on both BM and PB CD34⁺ cells.

Assessment of LTC-IC. The number of LTC-IC at week 5 of culture was 48.2 ± 35 (SEM) and 62.5 ± 54 (SEM) per 10^4 CD34⁺ cells in PB and BM, respectively (Table 2). Although there was a trend toward higher numbers of LTC-IC in BM samples, this observation did not achieve statistical significance. Overall, we harvested from 16 donors a mean number of 39,883 LTC-IC/kg of recipient body weight (range, 1,800 to 232,058) collected over two daily procedures.

Ara-C assays. At day 5 of G-CSF treatment, the proportion of BM progenitor cells in S-phase was 34.6% ± 11% (SD), as compared with a baseline value of 25.5% ± 12% (SD; $P < .05$; Table 3). As expected, G-CSF exerted its effect mainly on CFU-GM (52% ± 8% [SD] S-phase cells *v* 39.8% ± 10% [SD]; $P < .05$) rather than on BFU-E (Table 3). In sharp contrast, the number of circulating clonogenic precursors in S-phase from the same G-CSF–treated individuals was remarkably lower (4% ± 5% [SD]). This result was highly significant as compared with BM samples before and after G-CSF treatment ($P < .001$).

Short-term incubation with Ara-C was also used to determine the proportion of very primitive LTC-IC in S-phase. As in more mature progenitors, very few if any (1% ± 3% [SEM]) LTC-IC were in active synthesis of DNA, as compared with 21% ± 8% (SEM) in steady-state BM ($P < .001$; Table 2). Long-term exposure to Ara-C in the presence of growth factors was then used to evaluate the cycling status of LTC-IC.[14] We found that 67% ± 10% (SEM) of very primitive progenitor cells are recruited in cell cycle, with no statistical difference with BM samples (Table 2).

Cell-cycle studies. The experiments reported above show that the great majority of PB CD34⁺ cells are not in S-phase. However, they readily respond to CSFs and progress to mitosis, giving rise to a large number of colonies. Moreover, PB LTC-IC are inhibited by long-term incubation with Ara-C, suggesting that they progress through the cell

Table 2. Content and Kinetic Status of LTC-IC From G-CSF–Mobilized PB CD34⁺ Cells Compared With Steady-State BM Samples

Source of Cells	LTC-IC (×10⁴ CD34⁺ cells)	% S-Phase	% Cycling
PB	48.2 ± 35 (1.2-152)	1 ± 3	67 ± 10
BM	62.5 ± 54 (3.9-365)	21 ± 8*	75 ± 6

The number of LTC-IC was evaluated after 5 weeks of liquid culture as described in the Materials and Methods. To determine the percentage of S-phase and cycling LTC-IC, the CD34⁺ cell suspension was incubated with ARA-C for 1 hour and 16 hours, respectively, before the establishment of liquid culture. The results are expressed as the mean ± SEM (range) of 16 and 10 different experiments from PB and BM samples, respectively.

* Statistically significant compared with PB LTC-IC ($P < .001$).

Table 3. Percentage of S-Phase CD34+ Cells Purified From the BM and the PB of Normal Subjects Receiving rhG-CSF

Source of Cells	Pretreatment (%)			Posttreatment (%)		
	CFU-GM	BFU-E	Total	CFU-GM	BFU-E	Total
PB	ND	ND	ND	8.2 ± 6.2	3.1 ± 4	4 ± 5
BM	39.8 ± 10*†	17 ± 9†	25.5 ± 12†	52 ± 8*†	20 ± 12†	34.6 ± 11*†

The results are expressed as the mean ± SD of 16 and 10 different experiments from PB and BM samples, respectively. S-phase progenitors were evaluated before and after rhG-CSF treatment by the Ara-C suicide test.

Abbreviation: ND, not done.

* Statistically significant ($P < .05$) compared with pretreatment values.

† Statistically significant ($P < .05$) compared with PB CD34+ cells.

cycle. Thus, we posed the question as to whether, rather than being deeply quiescent in G_0-phase, circulating CD34+ cells might actually be cycling in G_1-phase. To test this hypothesis, we determined the cell-cycle characteristics of BM and PB CD34+ cells by the AO flow cytometric technique. The mean results are shown in Table 4 and confirm the negligible proportion of PB CD34+ cells in S- and G_2M-phase as compared with their BM counterparts. By contrast, the majority of PB hematopoietic progenitor cells was found in G_1-phase and their percentage was significantly higher than that reported in BM samples before and after G-CSF administration (80% ± 5% [SEM] *v* 61.9% ± 6% [SEM] and 48% ± 4% [SEM], respectively; $P < .05$). Notably, the number of PB CD34+ cells in G_0-phase was similar to that of BM samples. Moreover, combined DNA/RNA analysis showed that PB CD34+ cells have a significantly lower content of RNA than BM cells (Table 4). A representative example of simultaneous DNA/RNA analysis of PB and BM cells from the same healthy donor is presented in Fig 3.

We also used the AO staining to evaluate cellular apoptosis in the study samples (Table 4). A small but consistent and statistically significant difference between BM and PB cells was found (2.4% ± 0.5% [SEM] of circulating CD34+ cells were apoptotic compared with 3.7% ± 1% [SEM] in steady-state BM; $P < .05$). BM CD34+ cells assayed at day 5 of G-CSF administration showed the same percentage of apoptotic CD34+ cells as mobilized cells, suggesting that the effect on programmed cell death is due to G-CSF by itself rather than to a change of compartment (ie, mobilization into PB).

DISCUSSION

G-CSF has been shown to mobilize into PB cells with the capacity to reconstitute allogeneic hematopoiesis after a myeloablative conditioning regimen in both a murine model[19] and, more recently, in humans.[1-3] Transplantation of allogeneic PBSC has also resulted in a more rapid hematopoietic recovery as compared with marrow grafts.[1] Whether these effects are solely explained by quantitative changes in the numbers of different types of transplanted progenitors or whether the mobilization procedures induce relevant qualitative changes in the proliferative and differentiative behavior of both committed and primitive precursors is still a matter of investigation. Previous reports have suggested that circulating progenitor cells might be phenotypically and functionally distinct from those in the BM.[4,5,20] For instance, a somewhat surprising finding was that few committed PB precursors were in S-phase, whereas their BM counterparts were actively proliferating upon G-CSF priming.[5,20] It is a well-recognized fact that transplantation of mobilized stem cells results in a more rapid recovery of hematopoiesis than does reinfusion of BM cells. Moreover, mobilized CD34+ cells have generally shown a higher efficiency of retroviral vectors infection, requiring cell cycling for integration, as compared with marrow cells.

To better define the functional and kinetic characteristics of the human stem progenitor cells pool mobilized into PB by G-CSF, we determined (1) the frequency of CFU-C among mobilized CD34+ cells and their trilineage proliferative response to rhCSFs, (2) the content of very primitive LTC-IC, and (3) the cell-cycle distribution of PB CD34+ cells, including both clonogenic cells and LTC-IC, and the effects of G-CSF treatment on prevention or induction of apoptosis on hematopoietic stem cells. The results were then compared with those observed on BM CD34+ cells assessed before and during G-CSF administration. Notably, we studied cell populations that were virtually pure in CD34+ cells (mean value, >95%). In addition, the overall recovery of CD34+

Table 4. Cell-Cycle Distribution of CD34+ Cells

Source of Cells	% G_0	% G_1	% S	% G_2M	RNA-IG_1	% APO
BM (D0)	22.8 ± 7	61.9 ± 6	13.8 ± 2	1.7 ± 0.5	20.3 ± 0.7	3.7 ± 1
BM (D5)	20.3 ± 10	48 ± 4*	26 ± 4*	3 ± 1*	21.6 ± 5	2 ± 1*
PB (D5)	19.2 ± 4	80 ± 5*†	0.6 ± 0.3*†	0.1 ± 0.1*†	16.3 ± 0.5*†	2.4 ± 0.5*

The results are expressed as the mean ± SEM of 12 different experiments. RNA-IG_1 defines the RNA content of G_1 cells and it is determined as the ratio of the mean of RNA content of G_1 cells of study samples times 10, divided by the median RNA content of control lymphocytes.

Abbreviation: APO, apoptotic.

* Statistically significant versus baseline BM (D0) samples.

† Statistically significant versus day-5 BM (D5) samples.

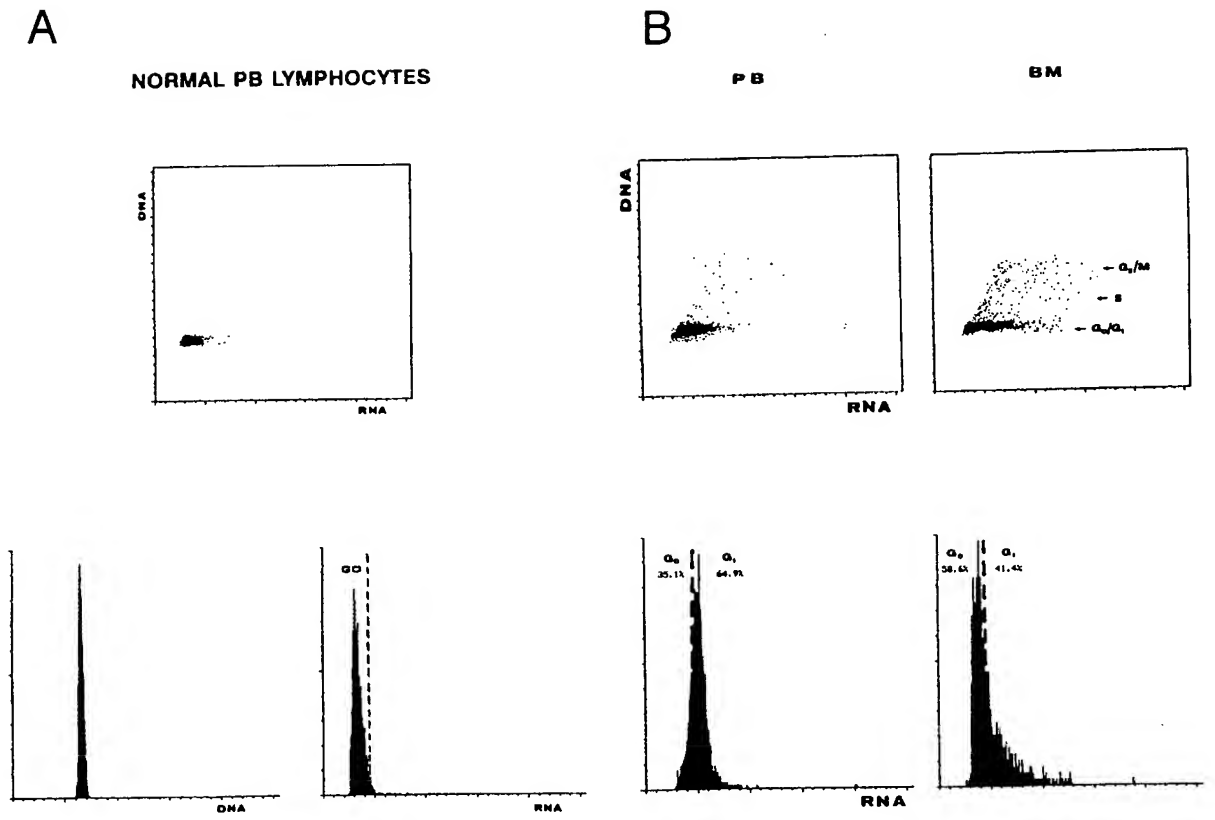


Fig 3. Combined DNA/RNA analysis of normal PB lymphocytes (A; control) and primed PB and steady-state BM CD34⁺ cells from the same healthy donor (B). All PB CD34⁺ cells were found in G_0/G_1 with 64.9% of cells being in G_1-phase with a low RNA content. Conversely, cell-cycle distribution of BM sample is as follows: G_0, 51.8%; G_1, 36.6%; S, 10.4%; and G_2M, 1.2%. The numerical results reported in the lower panels of the figure represent the sum of PB and BM cells found in G_0 + G_1-phase, normalized to 100%.

cells after positive selection was always greater than 85%; thus, the results reported here may be truly representative of the whole progenitor stem cells compartment. It should also be pointed out that, in contrast to previous studies, we did not compare our PBSC donors with random BM donors because of potential bias deriving from donor-to-donor variability with respect to progenitor cell clonogenic efficiency, LTC-IC content, and in vitro response to CSFs. Therefore, we studied highly purified BM and PB CD34⁺ cells from the same healthy individuals.

In our study, G-CSF–primed CD34⁺ cells showed the same clonogenic efficiency as their steady-state BM counterparts when stimulated with a conditioned medium. This is in agreement with previous data generated in a murine model[5] but not in humans.[4] To further investigate the proliferative potential of mobilized CD34⁺ cells, we assessed their trilineage colony-forming capacity in response to single growth factors or combinations of CSF selected for their ability to stimulate mature and more primitive hematopoietic progenitor cells, respectively.[21,22] Colony formation of CD34⁺ megakaryocyte progenitors (CFU-MK) collected from PB was not significantly different from that of BM CD34⁺ cells upon IL-3/GM-CSF and IL-3/IL-6/Epo stimulation. Circulating myeloid and erythroid CFU-C derived from CD34⁺ cells showed the same pattern of response to CSFs as BM cells, in line with reports by others (reviewed in Metcalf[21] and Ogawa[22]) and by our own group.[23] An early acting growth factor (ie, SCF) augmented both the size (data not shown) and the number of colonies stimulated by IL-3 and G-CSF (Fig 2). However, mobilized CD34⁺ cells showed a significantly higher clonogenic efficiency in response to cytokines other than G-CSF (ie, IL-3 and SCF) used alone and in combination. This finding may not be specific to mobilized progenitor cells, because we have already shown that priming with G-CSF increases the proliferative response of BM CD34⁺ cells to additional CSFs, including IL-3 and GM-CSF.[11] Therefore, this property of hematopoietic cells seems to be directly due to G-CSF treatment rather than to mobilization into PB.

We then performed comparative studies to further address the issue of kinetic changes of PB and BM CD34⁺ cells upon G-CSF treatment. In line with the findings of Roberts and Metcalf,[5] we documented the low percentage of mobilized CD34⁺ cells in S- and G_2M-phase through the use of two different assays. However, studies of cell-cycle distribution showed that the vast majority of circulating CD34⁺ cells are actually cycling, being in G_1-phase. Taken together, these data extend to normal PBSC donors previous observations

from nonhuman primates on cell-cycle status and response to CSF of cytokine-mobilized CD34+ cells.[20] Similar to the present study, the vast majority of circulating CD34+ cells were not found to be in active DNA synthesis. However, a greater proportion of PB hematopoietic cells entered the S/G_2M-phase within 72 hours after IL-6 and SCF stimulation.[20] Therefore, our results support the view that, rather than being deeply quiescent (G_0), as previously suggested by the low CD71 expression and the Rhodamine 123^{dull} status,[24] many of the circulating progenitors are in G_1 phase and might readily enter S-phase, particulary if exposed to certain growth factors.[20,25] The PB G_1 cells had a lower RNA content than BM cells and promptly proliferated in response to CSFs. This may indicate that the critical level of chromatin decondensation and nuclei accumulation of nonhistone proteins and RNA before DNA synthesis initiation is reached at a subthreshold level of RNA and protein content, thus resulting in a faster progression through cell cycle. The activity of G-CSF in the competence progression framework of the cell cycle may be similar to that of other early acting growth factors (ie, IL-11 and SCF) that have recently been shown to shorten G_1-phase.[26] The cycling status and the high response in vitro to growth factors may also explain why mobilized PB CD34+ cells represent an optimal target for efficient retroviral infection.[27] In addition, through kinetic analysis we have been able to show that G-CSF significantly protects circulating CD34+ cells from apoptosis, although this effect was not restricted to mobilized hematopoietic progenitor cells and was actually also observed on BM CD34+ cells. Thus, at least part of the response to G-CSF can be said to have been due to the increased survival of mobilized precursor populations.[28]

A further set of experiments was then designed to investigate the frequency and the cycling status of the most primitive subset of hematopoietic progenitor cells (LTC-IC) functionally identified for their capacity to generate CFU-C after 5 weeks of liquid culture. LTC-IC have recently been proposed as candidate long-term marrow repopulating cells,[8] and their presence in PB has been reported after mobilization protocols in cancer patients.[12] However, neither the level of LTC-IC nor their kinetic status has been evaluated in PBSC collections from normal donors intended for allogeneic transplantation. The assessment of cell-cycle characteristics of putative hematopoietic stem cells is important in the setting of allograft. In fact, competitive transplant studies in irradiated host mice have recently shown a defective long-term repopulating activity of early BM cells induced to S-phase by cytokines.[29] Moreover, there is increasing interest in determining the kinetic status of very primitive subfractions of CD34+ cells to improve our capacity to expand hematopoietic stem cells and use viral vectors. The results presented here do not show a significant difference in the frequency of LTC-IC between steady-state BM and primed PB CD34+ cells derived from the same normal subjects. Similar observations have been reported in cancer patients treated with G-CSF for 5 to 7 days for mobilization of autologous PBSC,[30] and this represents an approximately 100-fold increase compared with steady-state PB.[31] We observed a substantial interindividual variation with no relationship to age, sex, and white blood cell count (data not shown). Because PBSC grafts have been shown to contain 3^{32} to 5 (R.M.L., unpublished observations) more CD34+ cells than BM harvests, a similar increase may be assumed for LTC-IC. So far, 10 of our 16 patients have successfully received transplants after thawing PBSC collections, with a mean number of 26,723 PB LTC-IC/kg of recipient body weight (range, 1,800 to 113,739; collected over 2 daily procedures). These figures compare favorably with the number of LTC-IC per kilogram reported for autologous PBSC collections after G-CSF treatment (670 ± 300 [SEM] per single leukapheresis) and allogeneic BM grafts.[12] As with the PB committed progenitor cells, very few if any primitive hematopoietic cells were shown to be in S-phase, whereas 21% ± 0.8 % (SEM) of BM LTC-IC were inhibited by Ara-C ($P < .02$). However, by exposing CD34+ cells to Ara-C for 16 to 18 hours in the presence of appropriate CSFs,[14] we showed that about two-thirds of LTC-IC are in cell-cycle with no difference with baseline BM samples. Recently, Ponchio et al[14] have found that more than 80% of PB LTC-IC are quiescent under steady-state conditions, whereas 85% ± 5% (SEM) of BM LTC-IC are actively cycling. Thus, G-CSF seems to induce the mobilization of a large number of very primitive hematopoietic progenitor cells that share the same kinetic profile of BM cells.

In summary, our results indicate that CD34+ cells mobilized into PB of normal donors by glycosylated G-CSF (lenograstim) can be considered equivalent to their BM counterparts, on a cell per cell basis, in their content of both committed and very primitive hematopoietic progenitors capable of repopulating the hematopoietic tissue. Therefore, given their higher cellularity, PBSC collections should contain a substantially greater number of early hematopoietic cells than BM harvests. Kinetic and functional analysis of circulating CD34+ cells, studied at various stages of differentiation, shows that the majority of these cell populations are actually cycling, contain a very low number of apoptotic elements, and show an increased proliferative response to CSF stimulation as compared with steady-state BM precursors. However, comparative studies on BM cells before and during G-CSF treatment showed that some of these differences appear to be related directly to G-CSF administration rather than to change of compartment. These studies may have a major clinical significance in view of the increasing evidence that transplantation of allogeneic PBSC represents a feasible alternative to conventional BM graft.

REFERENCES

1. Korbling M, Przepiorka D, Huh YO, Engel H, van Bisen K, Giralt S, Andersson B, Kleine HD, Seong D, Deisseroth AB, Andreeff M, Champlin R: Allogeneic blood stem cell transplantation for refractory leukemia and lymphoma: Potential advantage of blood over marrow allografts. Blood 85:1659, 1995

2. Schmitz N, Dreger P, Suttorp M, Rohwedder EB, Haferlach T, Loffler H, Hunter A, Russel NH: Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). Blood 85:1666, 1995

3. Bensinger WI, Weaver CH, Appelbaum FR, Rowley S, Demirer T, Sanders J, Storb R, Buckner CD: Transplantation of alloge-

neic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor. Blood 85:1655, 1995

4. Tjonnfjord GE, Steen R, Evensen SA, Thorsby E, Egeland T: Characterization of CD34$^+$ peripheral blood cells from healthy adults mobilized by recombinant human granulocyte colony-stimulating factor. Blood 84:2795, 1994

5. Roberts AW, Metcalf D: Noncycling state of peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor and other cytokines. Blood 86:1600, 1995

6. Korbling M, HuhYO, Durett A, Mirza N, Miller P, Engel H, Anderlini P, van Besien K, Andreff M, Przepiorka D, Deisseroth AB, Champlin RE: Allogeneic blood stem cell transplantation: Peripheralization and yield of donor-derived primitive hematopoietic progenitor cells (CD34$^+$ Thy-1^{dim}) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. Blood 86:2842, 1995

7. Leitner A, Strobl H, Fischmeister G, Kurz M, Romanakis K, Haas OA, Printz D, Buchinger P, Bauer S, Gadner H, Fritsch G: Lack of DNA synthesis among CD 34+ cells in cord blood and in cytokine-mobilized blood. Br J Haematol 92:255, 1996

8. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM: Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74:1563, 1989

9. Lemoli RM, Fortuna A, Motta MR, Rizzi S, Giudice V, Nannetti A, Martinelli G, Cavo M, Amabile M, Mangianti S, Fogli M, Conte R, Tura S: Concomitant mobilization of plasma cells and hematopoietic progenitors into peripheral blood of multiple myeloma patients: Positive selection and transplantation of enriched CD34$^+$ cells to remove circulating tumor cells. Blood 87:1625, 1996

10. Miltenyi S, Guth S, Radbruch A, Pfluger E, Thiel A: Isolation of CD 34+ hematopoietic progenitor cells by high gradient magnetic cell sorting (MACS), in Wunder E, Sovalat H, Henon PR, Serke S (eds): Hematopoietic Stem Cells. The Mulhouse Manual. Dayton, OH, Alpha Med, 1994, p 201

11. Lemoli RM, Fortuna A, Fogli M, Gherlinzoni F, Rosti G, Catani L, Gozzetti A, Miggiano MC, Tura S: Proliferative response of human marrow myeloid progenitor cells to in vivo treatment with granulocyte colony-stimulating factor alone and in combination with interleukin-3 after autologous bone marrow transplantation. Exp Hematol 23:1520, 1995

12. Sutherland HJ, Eaves CJ, Lansdorp PM, Phillips GL, Hogge DE: Kinetics of committed and primitive blood progenitor mobilization after chemotherapy and growth factor treatment and their use in autotransplants. Blood 83:3808, 1994

13. Sutherland HJ, Eaves CJ, Lansdorp PM, Thacker JD, Hogge DE: Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. Blood 78:666, 1991

14. Ponchio L, Conneally E, Eaves CJ: Quantitation of the quiescent fraction of long-term culture-initiating cells in normal human blood and marrow and the kinetics of their growth factor-stimulated entry into S-phase in vitro. Blood 86:3314, 1995

15. Lemoli RM, Fortuna A, Tafuri A, Fogli M, Amabile M, Grande A, Ricciardi MR, Petrucci MT, Bonsi L, Bagnara GP, Visani G, Martinelli G, Ferrari S, Tura S: Interleukin-9 (IL-9) stimulates the proliferation of human myeloid leukemic cells. Blood 87:3852, 1996

16. Tafuri A, Lemoli RM, Chen R, Gulati SC, Clarkson BD, Andreeff M: Combination of hematopoietic growth factors containing IL-3 induce acute myeloid leukemia cells sensitization to cycle specific and cycle non-specific drugs. Leukemia 5:749, 1994

17. Andreeff M, Assing G, Cirrincione C: Prognostic value of DNA/RNA flow cytometry in myeloblastic and lymphoblastic leukemia in adults: RNA content and S-phase predict remission duration and survival in multivariate analysis. Ann NY Acad Sci 406:387, 1984

18. Darzynkiewicz Z, Bruno S, Del Bino G, Gorezyca W, Hotz MA, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. Cytometry 13:795, 1992

19. Molineux G, Pojda Z, Hampson IN, Lord BI, Dexter TM: Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. Blood 76:2153, 1990

20. Donahue RE, Kirby MR, Metzger ME, Agricola BA, Sellers SE, Cullis HM: Peripheral blood CD34$^+$ cells differ from bone marrow CD34$^+$ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor. Blood 87:1644, 1996

21. Metcalf D: Hematopoietic regulators: Redundancy or subtlety? Blood 82:3513, 1993

22. Ogawa M: Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844, 1993

23. Lemoli RM, Fogli M, Fortuna A, Motta MR, Rizzi S, Benini C, Tura S: Interleukin-11 stimulates the proliferation of human hematopoietic CD 34+ and CD 34+CD33-DR− cells and synergizes with stem cell factor, interleukin-3, and granulocyte-macrophage colony-stimulating factor. Exp Hematol 21:1668, 1993

24. To LB, Haylock DN, Dowse T, Simmons PJ, Trimboli S, Ashman LK, Juttner CA: A comparative study of the phenotype and proliferative capacity of peripheral blood (PB) CD34$^+$ cells mobilized by four different protocols and those of steady-state PB and bone marrow CD34$^+$ cells. Blood 84:2930, 1994

25. Standen G, Philip MA, Fletcher J: The proliferative state of granulocytic progenitor cells in human blood and marrow. Cell Tissue Kinet 13:105, 1980

26. Tanaka R, Katayama N, Ohishi K, Mahmud N, Itoh R, Tanaka Y, Komada Y, Minami N, Sakurai M, Shirakawa S, Shiku H: Accelerated cell-cycling of hematopoietic progenitor cells by growth factors. Blood 86:73, 1995

27. Bregni M, Magni M, Siena S, Di Nicola M, Bonadonna G, Gianni AM: Human peripheral blood hematopoietic progenitors are optimal targets of retroviral-mediated gene transfer. Blood 80:1418, 1992

28. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR: Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76, 1990

29. Peters SO, Kittler ELW, Ramshaw HS, Quesenberry PJ: Ex-vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11 and stem cell factor leads to impaired engraftment in irradiated host. Blood 87:30, 1996

30. Baumann I, Testa NG, Lange C, de Wynter E, Luft T, Dexter TM, van Hoef MEHM, Howell A: Haemopoietic cells mobilised into the circulation by lenograstim as alternative to bone marrow for allogeneic transplants. Lancet 341:369, 1993

31. Udomsakdi C, Lansdorp PM, Hogge DE, Reid DS, Eaves AC, Eaves CJ: Characterization of primitive hematopoietic cells in normal human peripheral blood. Blood 80:2513, 1992

32. Dreger P, Haferlach T, Eckstein V, Jacobs S, Suttorp M, Loffler H, Muller-Ruchholtz W, Schmitz N: G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: Safety, kinetics of mobilization, and composition of the graft. Br J Haematol 87:609, 1994

Synergistic Effect of FLT-3 Ligand on the Granulocyte Colony-Stimulating Factor–Induced Mobilization of Hematopoietic Stem Cells and Progenitor Cells Into Blood in Mice

By Yoshikazu Sudo, Chihiro Shimazaki, Eishi Ashihara, Takehisa Kikuta, Hideyo Hirai, Toshiya Sumikuma, Noboru Yamagata, Hideo Goto, Tohru Inaba, Naohisa Fujita, and Masao Nakagawa

We have previously shown that FLT-3 ligand (FL) mobilizes murine hematopoietic primitive and committed progenitor cells into blood dose-dependently. Whether FL also acts synergistically with granulocyte colony-stimulating factor (G-CSF) to induce such mobilization has now been investigated. Five- to 6-week-old C57BL/6J mice were injected subcutaneously with recombinant human G-CSF (250 μg/kg), Chinese hamster ovarian cell-derived FL (20 μg/kg), or both cytokines daily for 5 days. The number of colony-forming cells (CFCs) in peripheral blood increased approximately 2-, 21-, or 480-fold after administration of FL, G-CSF, or the two cytokines together, respectively, for 5 days. The number of CFCs in bone marrow decreased after 3 days but was increased approximately twofold after 5 days of treatment with G-CSF. The number of CFCs in the bone marrow of mice treated with both FL and G-CSF showed a 3.4-fold increase after 3 days and subsequently decreased to below control values. The number of CFCs in spleen was increased 24.2- and 93.7-fold after 5 days of treatment with G-CSF alone or in combination with FL, respectively. The number of colony-forming unit-spleen (CFU-S) (day 12) in peripheral blood was increased 13.2-fold by G-CSF alone and 182-fold by G-CSF and FL used together after 5 days of treatment. Finally, the number of preCFU-S mobilized into peripheral blood was also increased by the administration of FL and G-CSF. These observations show that FL synergistically enhances the G-CSF–induced mobilization of hematopoietic stem cells and progenitor cells into blood in mice, and that this combination of growth factors may prove useful for obtaining such cells in humans for transplantation.

SMALL NUMBERS of hematopoietic stem cells and progenitor cells are present in the peripheral blood (PB) of mice, dogs, and humans,[1-4] and large numbers of these cells are mobilized into blood by cytotoxic agents,[5] various growth factors,[6-8] or combinations of these.[9,10] The FLT-3 ligand (FL) is a ligand for the FLT3/FLK2 tyrosine kinase receptor expressed on hematopoietic stem cells[11,12] and stimulates the proliferation of hematopoietic stem cells and primitive progenitor cells in combination with other growth factors.[13-15] We have previously shown that FL can mobilize murine hematopoietic primitive and committed progenitor cells into the blood.[16] Given the potential clinical application of this molecule in the collection of allogeneic PB stem cells (PBSCs) from healthy donors for transplantation, it is important to establish a potent, nontoxic dosage regimen. Recently, granulocyte colony-stimulating factor (G-CSF) has been investigationally applied to mobilize PBSCs for allogeneic transplantation[17-19]; however, the use of G-CSF in high doses is associated with adverse effects such as general fatigue and bone pain.[20]

We have now investigated whether FL and G-CSF exhibit synergistic effects on the mobilization of PBSCs. We administered FL and G-CSF, alone or in combination, to mice and then assayed for the presence of colony-forming cells (CFCs) in the PB, bone marrow (BM), and spleen, as well as for colony-forming unit-spleen (CFU-S) and preCFU-S in the PB.

From the Second Department of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Submitted June 10, 1996; accepted December 16, 1996.

Address reprint requests to Eishi Ashihara, MD, Second Department of Medicine, Kyoto Prefectural University of Medicine, 465 Kawaramachi Hirokoji, Kamigyoku, Kyoto 602, Japan.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/97/8909-0014$3.00/0

MATERIALS AND METHODS

Mice

Five- to 6-week-old C57BL/6J mice, with body weight of 20 to 25 g, were obtained from Clea Japan (Osaka, Japan) and maintained in our animal facility.

Growth Factors

Chinese hamster ovarian (CHO) cell-derived FL was kindly provided by Dr Stewart Lyman (Immunex, Seattle, WA). Recombinant mouse (rm) stem cell factor (SCF), rm granulocyte-macrophage CSF (GM-CSF), and rm interleukin-3 (IL-3), as well as recombinant human (rh) G-CSF and rh erythropoietin (Epo) were kindly provided by Kirin Brewery (Tokyo, Japan).

Cell Harvesting

Mice were injected subcutaneously with rh G-CSF at a dose of 250 μg/kg alone or in combination with CHO cell-derived FL at a dose of 20 μg/kg for 5 days. PB was collected before and 3, 5, 7, and 9 days after the onset of treatment, and the number of white blood cells (WBCs) was determined. For all mice, 10 or 15 minutes after the injection of heparin (50 to 100 U), PB was collected from the orbital plexus and pooled before and 3, 5, 7, and 9 days after the onset of treatment. At each time point, one mouse in each group was killed by cervical dislocation, and both tibiae, both femora, and the spleen were dissected. BM cell suspensions were obtained by flushing the bones with 1 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 1% bovine serum albumin (BSA) (Sigma, St Louis, MO). Spleen cell suspensions were prepared by mincing the tissue with scissors, passing it through a 21-gauge needle, and then filtering through a 70-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). PB, BM, and spleen cell suspensions were layered over Ficoll-Metrizoate (Lymphoprep; Nakarai Tesque, Kyoto, Japan) and centrifuged at 1,700 rpm for 30 minutes at 4°C. Cells at the interface were removed and washed twice with phosphate-buffered saline containing 1% BSA. After the last wash, the cell pellet was suspended in IMDM containing 1% BSA.

CX1

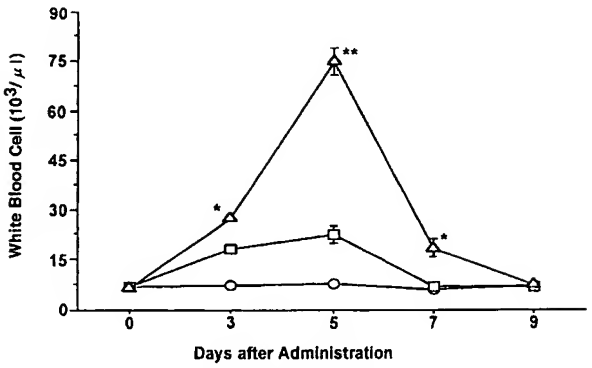


Fig 1. Time course of changes in the mean ± SE of total WBCs during the administration of FL at 20 μg/kg (○), G-CSF at 250 μg/kg (□), and FL plus G-CSF (△) for 5 days. Points that differ significantly from data for G-CSF administration on the same day are marked: **$P < .005$, *$P < .05$.

Committed Progenitor Cell Assay

CFCs, including colony-forming unit GM (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-Mix, were estimated by the standard methylcellulose method. One-milliliter suspensions of 5×10^4 to 2×10^5 nucleated PB cells, 1 to 2×10^4 nucleated marrow cells, or 5×10^4 to 1×10^5 nucleated spleen cells were plated in triplicate or quadruplicate. Cultures were supplemented with 20% fetal calf serum (GIBCO, Gaithersburg, MD), 2% l-glutamine (Nissui, Tokyo, Japan), 5×10^{-5} M 2-mercaptoethanol (Nakarai Tesque, Kyoto, Japan), 1% iron-saturated transferrin (5×10^{-7} mol/L; Sigma, St Louis, MO), rmSCF (100 ng/mL), rmIL-3 (100 U/mL), rmGM-CSF (50 ng/mL), rhG-CSF (50 ng/mL), and rhEpo (2 U/mL). The plates were incubated for 7 days at 37°C in a fully humidified 5% CO_2-air atmosphere, and colonies containing more than 50 cells were scored using an inverted microscope.

CFU-S Assay

For each data point, three to four recipient mice were irradiated with 9-Gy x-rays to prevent the production of endogenous spleen colonies as reported previously.[16] Irradiated mice were injected intravenously via the tail vein with 1 to 3×10^5 nucleated blood cells within several hours after the completion of irradiation. The mice were killed by cervical dislocation 12 days later, and the spleen was excised and fixed in Tellyesniczky's fixative. The number of macroscopic spleen colonies was then scored.

PreCFU-S Assay

We investigated whether the combination of FL and G-CSF could mobilize preCFU-S into PB. A spleen from one of three mice that had been irradiated and injected with nucleated blood cells 12 days earlier was excised. Spleen cell suspensions were prepared as described above. After the number of CFU-S (day 12) in other spleens of recipient mice was scored, the single cell suspension was diluted and injected further into irradiated mice (0.3 to 1 colony per mouse). The number of daughter colonies (preCFU-S) was scored 12 days later. The number of colonies generated per CFU-S (day 12) was calculated as described previously,[21] and the number of preCFU-S was estimated as total number of preCFU-S per milliliter of PB.

Statistic analysis. Data are expressed as mean ± SE and were analyzed by Student's *t*-test. A P value of $< .05$ was considered statistically significant.

RESULTS

Effects of FL and G-CSF on Circulating WBCs

Administration of G-CSF at a dose of 250 μg/kg induced an approximately threefold increase in the total number of WBCs after 5 days of treatment as compared with the number present before treatment (Fig 1). FL at a dose of 20 μg/kg had no effect on the number of WBCs, whereas the combination of FL (20 μg/kg) and G-CSF (250 μg/kg) increased the number of these cells to a markedly greater extent than did G-CSF alone ($P < .005$). The percentage of neutrophils in the WBC fraction was increased slightly more by the combination of FL and G-CSF than by G-CSF alone. Immature cells, myelocytes and metamyelocytes, were also more increased in the PB of mice given FL and G-CSF (data not shown).

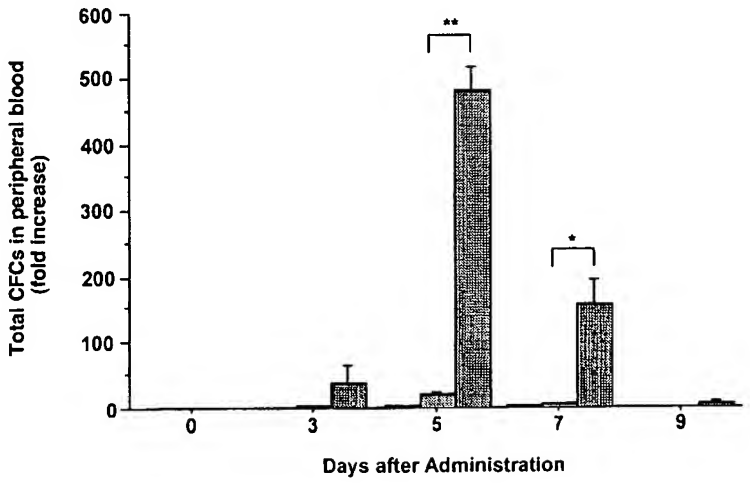


Fig 2. Changes in the number of total CFCs in the PB during the administration of FL at 20 μg/kg (□), G-CSF at 250 μg/kg (▨), and FL plus G-CSF (■) for 5 days. Data are mean ± SE and are expressed as fold increases relative to mice before treatment. Points that differ significantly from data for G-CSF administration on the same day are marked: **$P < .005$, *$P < .05$.

Table 1. Percentage of Various Lineages of CFCs Mobilized Into PB During Cytokine Treatment

Cytokine	Day	Percentage of CFCs			No. of Total CFCs in PB (/mL)
		CFU-GM	BFU-E	CFU-Mix	
	0	69.5 ± 2.7	21.0 ± 4.0	9.5 ± 3.1	220 ± 0
FL	3	52.0 ± 2.7	30.9 ± 1.2	17.1 ± 0.1	412 ± 29
(20 μg/kg)	5	56.6 ± 1.2	30.0 ± 0.5	13.4 ± 2.6	846 ± 35
G-CSF	3	94.1 ± 0.9	3.6 ± 1.7	2.3 ± 0.7	910 ± 68
(250 μg/kg)	5	94.5 ± 1.7	4.4 ± 1.5	1.1 ± 0.4	8,220 ± 31
FL + G-CSF	3	96.3 ± 0.4	2.9 ± 0.4	0.8 ± 0.2	15,929 ± 624
	5	94.7 ± 0.6	4.3 ± 0.3	0.1 ± 0.4	109,277 ± 5,875

Data are shown as the mean ± SD of percentage of colonies derived from triplicate or quadruplicate cultures on samples at each time point.

Effects of FL and G-CSF on the Mobilization of Committed Progenitor Cells Into the Circulation

The number of hematopoietic committed progenitor cells in the PB was increased 2.2- and 21.3-fold after 5 days of treatment with FL or G-CSF, respectively (Fig 2). In contrast, the combination of FL and G-CSF caused an approximately 480-fold increase in the total number of CFCs in PB at the same point, which was statistically significant as compared with those given the same dose of G-CSF alone ($P < .005$). Although various types of CFCs (CFU-GM, BFU-E, and CFU-Mix) were increased in blood, the administration of G-CSF alone or in combination with FL mobilized predominantly CFU-GM (Table 1).

Effects of FL and G-CSF on Committed Progenitor Cell Numbers in the BM and Spleen

The total number of CFCs in the BM (femora and tibiae) showed a maximal, 2.7-fold increase after 5 days of treatment with FL (Fig 3). The number of CFCs was decreased 3 days after and showed a twofold increase 5 days after the onset of treatment with G-CSF. In mice treated with both FL and G-CSF, the total number of CFCs in BM showed a maximal, 3.4-fold increase after 3 days and then decreased to below control values. The total number of CFCs in the spleen showed a 24.2-fold increase after 5 days of treatment with G-CSF (Fig 4), at which time the spleen was enlarged (Fig 5). The administration of both FL and G-CSF induced marked splenomegaly (Fig 5) and a 93.7-fold increase in the number of total CFCs in spleen 5 days after the onset of treatment (Fig 4) ($P < .05$).

Effects of FL and G-CSF on CFU-S (Day 12) in PB

The formation of spleen colonies in the irradiated mice injected with mobilized cells was also measured. Increases in the mean number of CFU-S (day 12) in the blood of mice injected with FL, G-CSF, and the combination of these two factors are plotted as fold increases (relative to control mice analyzed on the same day) in Fig 6. Administration of FL alone did not mobilize CFU-S (day 12) into the blood. Although G-CSF alone did cause an increase of 13.1-fold in CFU-S (day 12), the combination of G-CSF and FL caused an increase of 182-fold in PB CFU-S (day 12) 5 days after the onset of treatment (Fig 6) ($P < .005$).

Effects of FL and G-CSF on preCFU-S in PB

The mobilization of preCFU-S after administration of G-CSF alone or in combination with FL is shown in Table 2.

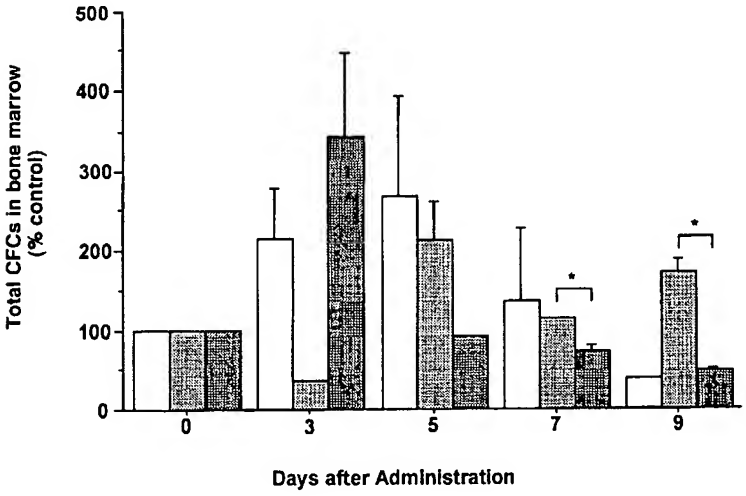


Fig 3. Changes in the number of total CFCs in the BM during the administration of FL at 20 μg/kg (□), G-CSF at 250 μg/kg (□), and FL plus G-CSF (■) for 5 days. Data are mean ± SE and are expressed as a percentage of total CFC numbers before cytokine administration. Points that differ significantly from data for G-CSF administration on the same day are marked: *$P < .05$.

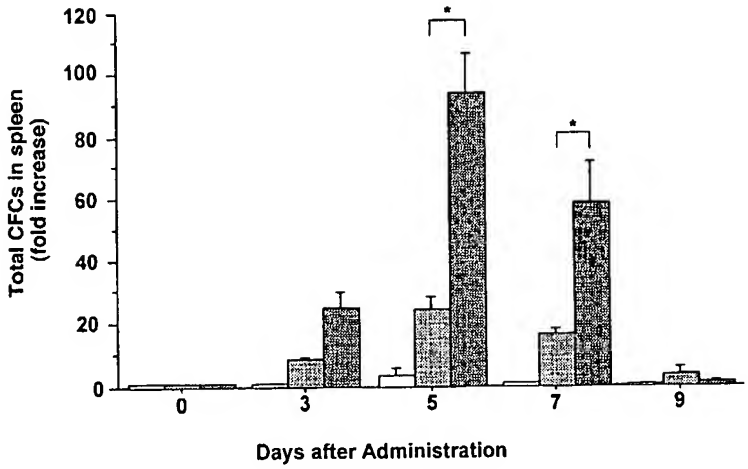


Fig 4. Changes in the number of total CFCs in the spleen during the administration of FL at 20 μg/kg (□), G-CSF at 250 μg/kg (▨), and FL plus G-CSF (■) for 5 days. Data are mean ± SE and are expressed as fold increases relative to mice before treatment. Points that differ significantly from data for G-CSF administration on the same day are marked: *$P < .05$.

While G-CSF alone induced a 315-fold increase in preCFU-S in the PB, the combination of FL and G-CSF induced a 1,048-fold increase in the number of preCFU-S in the blood 5 days after the onset of treatment. Thus, the combination of FL and G-CSF strikingly mobilized preCFU-S into the blood.

DISCUSSION

In a previous study we showed that FL can mobilize primitive and committed hematopoietic progenitor cells into the blood in a dose-dependent fashion and that the administration of FL alone at 20 μg/kg mobilizes only small numbers of primitive and committed hematopoietic progenitor cells into blood.[16] Therefore, we investigated whether a low dose of FL would have synergistic effects on the mobilizing effect obtained with G-CSF. In mice, FL synergistically enhances the leukocytosis by G-CSF. Moreover, the combination of FL and G-CSF increased the number of more immature granulocytes present in the PB than did either cytokine alone.

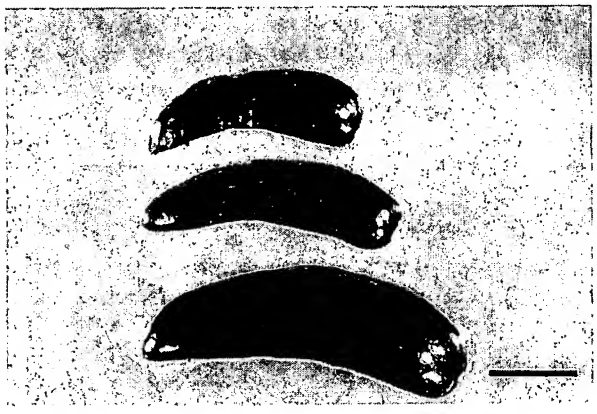


Fig 5. Spleens from a control mouse (top) and from mice treated for 5 days with G-CSF (250 μg/kg) alone (middle), or together with FL (20 μg/kg) (bottom). The bar indicates 5 mm.

Although the administration of FL at a low dose of 20 μg/kg did not mobilize CFCs or CFU-S into blood, it significantly potentiated the increase in the number of CFCs in PB and spleen as well as the number of CFU-S (day 12) in the PB induced by G-CSF. The administration of FL mobilized various lineages of CFC into the blood,[16,22] whereas approximately 94% of the CFCs mobilized either by G-CSF alone or by FL plus G-CSF were CFU-GM (Table 1). One possibility for this is that the dose of FL was too low to influence the types of CFCs mobilized by G-CSF treatment. Therefore, it would be interesting to study types of CFCs mobilized by higher doses of FL with G-CSF. The number of CFC in the BM was not significantly increased by the administration of FL or G-CSF either alone or in combination. The number of CFC in the BM was maximal after 3 days of treatment with both FL and G-CSF, and subsequently decreased through 9 days after the onset of treatment. In mice injected with G-CSF, the number of these cells was below control values and then increased to as much as twice control values 3 and 5 days, respectively, after the onset of treatment. A peak number of CFCs in the BM of mice treated with FL was apparent after 5 days.

These observations suggest that the mechanism of CFC mobilization by G-CSF plus FL differs from that induced by FL or G-CSF alone. Roberts and Metcalf[23] showed that hematopoietic progenitor cells in the BM first move to the PB or spleen and then proliferate in mice treated with G-CSF alone. We have previously shown that redistribution as well as in vivo expansion of hematopoietic progenitor cells appear to contribute to the mobilization of these cells induced by FL in mice[16] because the administration of FL alone led to a slight increase in the number of CFC in BM. It is likely that primitive and committed hematopoietic progenitor cells first proliferate more rapidly in the BM of mice treated with G-CSF plus FL than in that of mice treated with either cytokine alone, with the maximal number of HPCs in the BM apparent after 3 days of treatment. These cells then move into the PB and then into the spleen, resulting in maximal numbers being apparent at these sites after 5 days. The num-

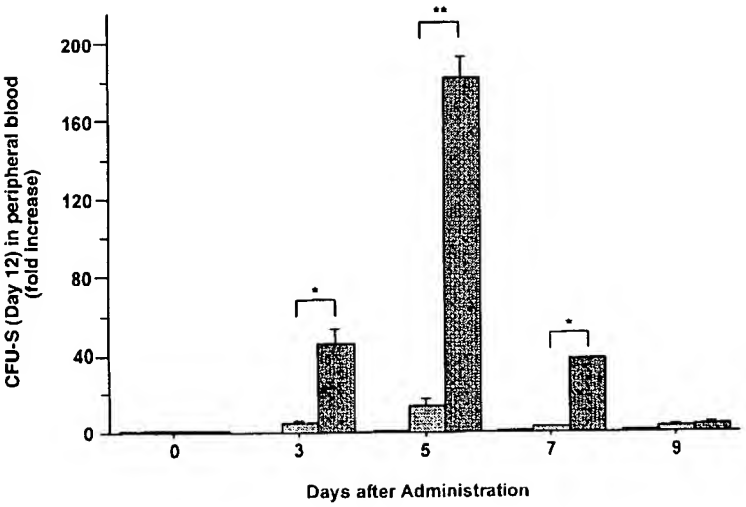


Fig 6. Changes in the number of CFU-S (day 12) during the administration of FL at 20 μg/kg (□), G-CSF at 250 μg/kg (□), and FL plus G-CSF (■) for 5 days. Data are expressed as mean ± SE fold increases relative to mice before treatment. Points that differ significantly from data for G-CSF administration on the same day are marked: **$P < .005$, *$P < .05$.

ber of CFC in the BM of mice treated with both FL and G-CSF decreased to below control values 7 and 9 days after the onset of treatment. One possible explanation for this finding is that the hematopoietic stem cells in the BM of mice treated with both these agents move into the PB or the spleen after hematopoietic stem cells proliferate and differentiate into progenitor cells more than these cells do in mice treated with either agent alone, resulting in a decrease of progenitor cells in the BM. It is therefore likely that the splenomegaly apparent in mice treated with FL and G-CSF results from the movement of the expanded progenitor cells from the BM to the spleen and from the further proliferation of these cells in the spleen.

We also examined the mobilization of preCFU-S into the blood. Whereas G-CSF mobilized preCFU-S, the administration of a low dose of FL potentiated the increase in the number of mobilized preCFU-S by G-CSF. Because preCFU-S may be closely related to long-term reconstituting cells, these findings suggest that FL synergistically enhances the G-CSF-induced mobilization of hematopoietic stem cells into the blood of mice.

Recently, SCF has also been shown to mobilize hematopoietic stem cells and progenitor cells into the PB of primates,[24] and clinical trials monitoring this effect of SCF alone or in combination with G-CSF are underway.[25-27] However, several subjects in these trials developed respiratory symptoms, including hoarseness, cough, and laryngospasm,[26] presumably because SCF induces the proliferation and maturation of mast cells.[28,29] Because FL does not affect the growth of mast cells or their degranulation,[30] its in vivo administration would be expected to induce fewer and less masked allergic reactions than SCF. The combination of FL with G-CSF may thus prove useful for harvesting PBSCs for transplantation. Further studies are warranted to determine whether FL with G-CSF could have synergistic effects on the mobilization of progenitors in humans.

Table 2. Changes in the Number of preCFU-S in PB on Day 5 After Administration of G-CSF Alone or in Combination With FL

Cytokine		PreCFU-S/CFU-S	PreCFU-S/mL of PB	Fold Increase
	Day 0	1.1 ± 1.1	18.2 ± 18.2	
G-CSF	Day 5	8 ± 0	5,747.6 ± 0	315.8
G-CSF + FL	Day 5	6.3 ± 1.6	19,089 ± 5,510	1,048.8

REFERENCES

1. Goodman JW, Hodgson GS: Evidence for stem cells in peripheral blood of mice. Blood 19:702, 1962

2. Cavuns JA, Scheer SC, Thomas ED, Ferrebee JW: The recovery of lethally irradiated dogs given infusions of autologous leukocytes preserved at −80°C. Blood 23:38, 1964

3. McCredie KB, Hersh EM, Freireich EJ: Cells capable of colony formation in the peripheral blood of man. Science 171:293, 1971

4. Barr RD, Whang-Peng J: Hematopoietic stem cells in human peripheral blood. Science 190:284, 1975

5. To LB, Shepperd KM, Haylock DN, Dyson PG, Charles P, Thorp DL, Dale BM, Dart GM, Roberts MM, Sage RE, Juttner CA: Single high doses of cyclophosphamide enable the collection of high numbers of hematopoietic stem cells from the peripheral blood. Exp Hematol 18:442, 1990

6. Molineux G, Pojda Z, Hampson IN, Lord BI, Dexter TM: Transplantation potential of peripheral blood stem cells induced by granulocyte colony-stimulating factor. Blood 76:2153, 1990

7. Ganser A, Lindermann A, Ottamann OG, Seipelt G, Hess U, Geissler G, Kanz L, Frisch J, Schulz G, Hermann F, Mertelsmann R, Hoelzer D: Sequential in vivo treatment with two recombinant human hematopoietic growth factors (interleukin-3 and granulocyte-macrophage colony-stimulating factor) as a new therapeutic modality to stimulate hematopoiesis: Results of a Phase I study. Blood 79:2583, 1992

8. Bodine DM, Seidel NE, Zsebo KM, Orlic D: In vivo administration of stem cell factor to mice increases the absolute number of pluripotent hematopoietic stem cells. Blood 82:445, 1993

9. Siena S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni M: Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: Enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood 74:1905, 1989

10. Shimazaki C, Oku N, Ashihara E, Okawa K, Goto H, Inaba T, Ito K, Fujita N, Tsuji H, Murakami S, Haruyama H, Nishio A, Nakagawa M: Collection of peripheral blood stem cells mobilized by high-dose Ara-C plus VP-16 or aclarubicin followed by recombinant human granulocyte-colony stimulating factor. Bone Marrow Transplant 10:341, 1992

11. Matthew W, Jordan CT, Wiegand GD, Pardoll D, Lemischka IR: A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 65:1143, 1991

12. Rosnet O, Schiff C, Pébusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F, Birnbaum D: Human *FLT 3/FLK 2* gene: cDNA cloning and expression in hematopoietic cells. Blood 82:1110, 1993

13. Lyman SD, James L, Bos TV, de Vries P, Brasel K, Gliniak B, Hollingsworth LT, Picha KS, McKenna HJ, Splett RR, Fletcher FA, Maraskovsky E, Farrah T, Foxworthe D, Williams DE, Beckmann P: Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: A proliferative factor for primitive hematopoietic cells. Cell 75:1157, 1993

14. Hudak S, Hunte B, Culpepper J, Menon S, Hannum C, Thompson-Snipe L, Rennick D: FLT3/FLK2 ligand promotes the growth of murine stem cells and the expansion of colony-forming cells and spleen colony-forming units. Blood 85:2747, 1995

15. Lyman SD, James L, Johnson L, Brasel K, de Vries P, Escobar SS, Downey H, Splett R, Beckmann MP, McKenna HJ: Cloning of the human homologue of the murine flt3 ligand: A growth factor for early hematopoietic progenitor cells. Blood 83:2795, 1994

16. Ashihara E, Shimazaki C, Sudo Y, Kikuta T, Hirai H, Sumikuma T, Yamagata N, Goto H, Inaba T, Fujita N, Nakagawa M: FLT-3 ligand mobilizes hematopoietic stem/progenitor cells into blood in mice. Exp Hematol 23:801a, 1995 (abstr)

17. Bensinger WI, Weaver CH, Appelbaum FR, Rowley S, Demirer T, Sanders J, Strob R, Bucker CD: Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor. Blood 85:1655, 1995

18. Schmitz N, Dreger P, Suttorp M, Rohweddeer EB, Haferlach T, Löffler H, Hunter A, Russell NH: Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). Blood 85:1666, 1995

19. Körbling M, Huh YO, Durett A, Mirza N, Miller P, Engel H, Anderlini P, van Besien K, Andreeff M, Prepiorka D, Deisseroth AB, Champlin R: Allogeneic blood stem cell transplantation: Peripheralization and yield of donor-derived primitive hematopoietic progenitor cells ($CD34^+$ $Thy\text{-}1^{dim}$) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. Blood 86:2842, 1995

20. Grigg AP, Roberts A, Raunow H, Houghton S, Layton JE, Boyd AW, McGrath KM, Maher D: Optimizing dose and scheduling of Filgrastim (granulocyte colony-stimulating factor) for mobilization and collection of peripheral blood progenitor cells in normal volunteers. Blood 86:4437, 1995

21. Drize N, Gan O, Zander A: Effect of recombinant human granulocyte colony-stimulating factor treatment of mice on spleen colony-forming unit number and self-renewal capacity. Exp Hematol 21:1289, 1993

22. Brasel K, McKenna HJ, Morrissey PJ, Charrier K, Morris AE, Lee CC, Williams DE, Luman SD: Hematopoietic effects of flts ligand in vivo in mice. Blood 88:2004, 1996

23. Roberts AW, Metcalf D: Granulocyte colony-stimulating factor induces selective elevations of progenitor cells in the peripheral blood of mice. Exp Hematol 22:1156, 1994

24. Andrews RG, Briddell R, Knitter GH, Opie T, Bronsden M, Myerson D, Appelbaum FR, McNiece IK: In vivo synergy between recombinant human stem cell factor and recombinant human granulocyte colony-stimulating factor in baboons: Enhanced circulation of progenitor cells. Blood 84:800, 1994

25. Gordon MS, Costa J, Sledge GW, Hayes D, Galli S, Hoffman R, Merica E, Rich W, Harkins B, McGuire B, Demetri G: Phase I trial of recombinant methionyl human stem cell factor (SCF) in women with locally advanced (IIIB) or cyclophosphamide (C) and doxorubicin (A). Exp Hematol 21:519a, 1993 (abstr)

26. Kurtzberg J, Meyers F, McGuire B, Crawford J: Mobilization of peripheral blood progenitor cells in patients given recombinant methionyl human stem cell factor. Proc Am Assoc Cancer Res 34:1257a, 1993 (abstr)

27. Glaspy J, LeMaistre CF, Lill M, Jones R, Moore A, Briddell, Menchaca D, Turner S, Shpall EJ: Dose-response of 7 day administration of recombinant methionyl human stem cell factor (SCF) in combination with filgrastim (G-CSF) for progenitor cell mobilization in patients with stage II-IV breast cancer. Blood 86:463a, 1995 (abstr, suppl 1)

28. Tsai M, Takeishi T, Thompson H, Langley KE, Zsebo KM, Metcalf DD, Geissler EN, Galli S: Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. Proc Natl Acad Sci USA 88:6382, 1991

29. Mitsui H, Furitsu T, Dvorak AM, Irani AA, Schwartz LB, Inagaki N, Takei M, Ishizaka K, Zsebo KM, Gillis S, Ishizaka T: Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand. Proc Natl Acad Sci USA 90:735, 1993

30. Lyman SD, Brasel K, Rousseau AM, Williams DE: The flt3 Ligand: A hematopoietic stem cell factor whose activities are distinct from steel factor, in Murphy MJ Jr (ed): Polyfunction of Hematopoietic Regulators, The Metcalf Forum. Dayton, OH, AlphaMed, 1994

Exhibit 5

Rapid Mobilization of Hematopoietic Progenitor Cells in Rhesus Monkeys by a Single Intravenous Injection of Interleukin-8

By L. Laterveer, I.J.D. Lindley, D.P.M. Heemskerk, J.A.J. Camps, E.K.J. Pauwels, R. Willemze, and W.E. Fibbe

Interleukin-8 (IL-8) is a chemoattractant cytokine involved in chemotaxis and activation of neutrophils. Because in vivo administration of IL-8 induces mobilization of hematopoietic stem cells in mice, we assessed the mobilizing properties of IL-8 in rhesus monkeys. Recombinant human IL-8 was administered as a single intravenous injection at doses of 10, 30, and 100 μg/kg to rhesus monkeys (age, 2 to 3 years; weight, 2.5 to 4.5 kg). Venous blood samples were obtained at time intervals ranging from 1 to 480 minutes after IL-8 administration. Cell counts, colony-forming unit-Mix assays, and fluorescence-activated cell sorter analysis were performed. Plasma was harvested to assess IL-8 levels. A time-controlled bolus intravenous injection of 100 μg IL-8 per kilogram of body weight resulted in peak IL-8 plasma levels up to 5 μg/mL. The calculated half-time life of free IL-8 was 9.9 ± 2.2 minutes. IL-8 injection resulted in instant neutropenia that was due to pulmonary sequestration, as shown using ^{99m}Tc-labeled leukocytes. Within 30 minutes after IL-8 injection, neutrophilia developed with counts up to 10-fold greater than baseline levels. The numbers of hematopoietic progenitor cells (HPCs) increased from 45 ± 48/mL to 1,382 ± 599/mL of blood at 30 minutes after injection of 100 μg IL-8 per kilogram of bodyweight (mean ± SD, n = 8). Individual animals showed 10- to 100-fold increase in numbers of circulating HPCs that returned to almost pretreatment values (92 ± 52 CFU/mL) at 240 minutes after the injection of IL-8. Immunophenotyping showed no significant changes in lymphocyte (sub)populations. A second bolus injection of IL-8 with an interval of 72 hours resulted in similar numbers of mobilized stem cells as observed after the first injection, showing that no tachyphylaxis had occurred. We conclude that IL-8 induces mobilization of HPCs from the bone marrow of rhesus monkeys in a rapid and reproducible fashion. Therefore, IL-8 may be a potentially useful cytokine in the setting of blood stem cell transplantation.

INTERLEUKIN-8 (IL-8) belongs to a family of proinflammatory molecules called chemokines.[1,2] It was previously known as neutrophil activating protein (NAP-1)[3-7] and is a member of the CXC chemokine subfamily that includes several related ligands involved in the activation and chemoattraction of neutrophils.[8] The genes encoding these peptides are clustered on the human chromosome 4q13-q21.[8] IL-8 is produced by a variety of cells, ie, monocytes, neutrophils, fibroblasts, endothelial cells, lung epithelial cells, mast cells, and keratinocytes,[3-15] in response to stimulation with lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-2, or IL-3.[16-19] The in vitro bioactivities of IL-8 include chemotaxis,[3-7] neutrophil activation, induction of release of metalloproteinases (ie, elastase, gelatinase-B, and β-glucuronidase), production of toxic metabolites in neutrophils,[4,5,20-22] stimulation of histamine release by basophils,[23,24] inhibition of neutrophil-endothelial interaction,[10,25] shedding of L-selectin,[25,26] upregulation of CD11b/CD18,[25,27] and transendothelial migration of neutrophils.[28]

In vivo, local administration of IL-8 induces a typical inflammatory response characterized by neutrophil margination and infiltration, plasma exudation, and angiogenesis.[7,29-33] Systemic treatment with IL-8 in monkeys, rabbits, and mice induces an initial neutropenia that is rapidly followed by granulocytosis.[29,34-36]

Previously, we reported the rapid mobilization of hematopoietic stem cells after a single injection of IL-8 in mice.[36] These stem cells had radioprotective capacity and long-term lymphomyeloid repopulating ability. Because IL-8 is relatively species-specific[37] and in view of its potential application in humans, we wished to study these effects of IL-8 in nonhuman primates. Up to now, no reports have been published on the mobilization of hematopoietic progenitor cells in primates by IL-8.

In this report, we show that a single intravenous injection of IL-8 in rhesus monkeys causes an immediate neutropenia due to sequestration of neutrophils in the lungs. This neutropenia is followed by a mean 30-fold increase in the numbers of circulating progenitor cells until, at several hours after injection, an absolute neutrophilia occurs. These progenitor cell mobilizing properties of IL-8 indicate its potential use in the setting of peripheral blood stem cell transplantation.

MATERIALS AND METHODS

Animals. Ten female rhesus monkeys (Macaca mulatta) bred at the TNO Primate Center (Rijswijk, The Netherlands) were used throughout this study. The monkeys were 2 to 3 years of age and weighed from 2.5 to 4.5 kg. The animals were free of intestinal parasites, herpes-B, simian T-cell leukemia virus (STLV), and simian immunodeficiency virus (SIV). Experiments were approved by the Ethical Committee on Animal Research. Some animals were used in three experiments with intervals of at least 4 weeks to certify that all hematologic parameters had returned to baseline levels.

IL-8. Human recombinant *Escherichia coli*-derived IL-8[20] was obtained from the laboratory of IJDL (Sandoz Forschungsinstitut, Vienna, Austria). IL-8 had no colony-stimulating activity, as reported previously[38] and as confirmed in our laboratory (unpublished

From the Laboratory of Experimental Hematology, Department of Hematology and the Department of Diagnostic Radiology and Nuclear Medicine, University Hospital Leiden, The Netherlands; and Sandoz Forschungsinstitut, Vienna, Austria.

Submitted June 15, 1995; accepted August 18, 1995.

Supported in part by the J.A. Cohen Institute for Radiobiology and Radioprotection and by the Dutch Cancer Foundation (NKB, Project Grant No. RUL 95-1091).

Address reprint requests to W.E. Fibbe, MD, PhD, Department of Hematology, University Hospital Leiden, Bldg-1, C2R, PO Box 9600, 2300 RC Leiden, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/96/8702-0020$3.00/0

CU1

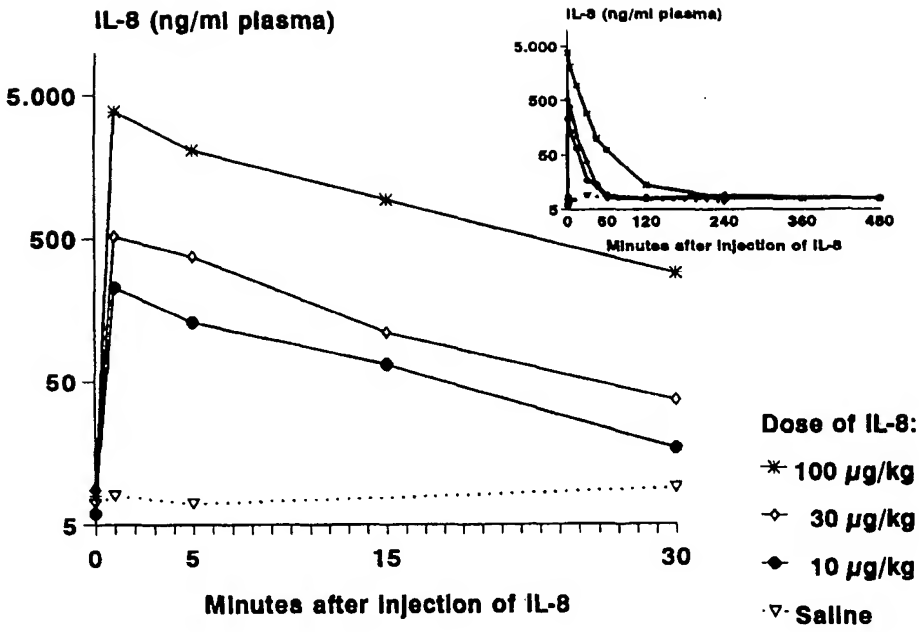


Fig 1. Effect of IL-8 on the levels of circulating IL-8. Saline or IL-8 (10, 30, and 100 µg/kg) was injected intravenously at t = 0. Results are expressed as the means of two (10 µg/kg), three (30 µg/kg), or four (100 µg/kg) experiments. The saline experiment was performed once. The inserted figure shows the plasma levels of IL-8 up to 480 minutes after injection.

observations, March and May 1991). The concentration of endotoxin was less then 0.05 EU/mL, as tested in the Limulus amoebocyte lysate assay. For in vivo experiments, IL-8 was diluted to the desired concentration in endotoxin-free phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and administered as a time-controlled (30 seconds) intravenous bolus injection.

Preparation of cell suspensions. Blood samples were taken by venous puncture at various intervals and total blood cell counts were performed on a Sysmex F1000 (Toa Medical Electronics Co LTD, Kobe, Japan). Manual differential counts were performed on May-Grunwald-Giemsa–stained blood films. Plasma was separated from the blood cells by centrifugation and the cell pellet was resuspended in an equal volume of RPMI 1640 containing 8 U/mL heparin and 50% (vol/vol) fetal bovine serum (FBS; GIBCO, Grand Island, NY). Erythrocytes were lysed by incubation with NH_4Cl-buffer for 10 minutes at 0°C. Leukocytes were washed two times with washing buffer (RPMI 1640 supplemented with 500 µg/mL penicillin, 250 µg/mL streptomycin, 8 U/mL heparin, and 2% [vol/vol] FBS). Cell numbers were counted and diluted to the desired concentration in Iscove's modified Dulbecco's medium (IMDM).

Colony-forming unit-Mix (CFU-Mix) cultures. Cells were cultured in 3.5-cm dishes in semisolid medium. One milliliter of medium consisted of 10 ng recombinant human (rhu) GM-CSF, 10 ng recombinant human granulocyte colony-stimulating factor (rhuG-CSF), 10 ng recombinant human stem cell factor (rhuSCF), 2 U recombinant human erythropoietin (rhuEPO), and 50 ng rhuIL-3, 10^{-5} mol/L β-mercaptoethanol, 500 ng transferrin, 1.1% methylcellulose, and 20% vol/vol human plasma in IMDM. Blood cell samples were plated in triplets at a concentration of 5×10^5 cells/mL. After 7 to 8 days of culture at 37°C, 5%CO_2 in a fully humidified atmosphere, the numbers of CFUs (defined as aggregates of >20 cells) were scored using an inverted microscope.

Imaging procedure. Twenty milliliters of venous blood was collected in citrate and erythrocytes were lysed as described above. After washing two times in sterile and pyrogen-free saline containing 2% autologous serum, leukocytes were centrifuged and the cell pellet was incubated for 10 minutes with 294 MBq technetium-99m (^{99m}Tc) exametazime (Ceretec; Amersham International, Amersham, UK). Cells were washed with 2% serum and the labeling efficiency was determined and found to be 49%. The cell pellet was resuspended in serum of the monkey and reinfused after sedating the monkey. Total body scintigrams (head to knees) were made with the monkey in the supine position on a gammacamera with a field of view of 35×50 cm (CGA-90B; Toshiba, Tokyo, Japan). Images were acquired every 20 seconds for a period of 70 minutes after injection. Starting at 22 minutes after injection of IL-8, blood samples were taken at several time intervals to determine blood cell counts. Time activity curves were generated over the total imaging period for the regions of interest (lungs and aorta).

Assay for IL-8. Plasma levels of IL-8 were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) with a commercially available IL-8 compact kit (CLB, Amsterdam, The Netherlands). Plasma was diluted from 1:100 up to 1:3,200. The lower detection limit of the assay in plasma was 6 to 10 ng/mL plasma. Half-life time values were calculated by nonlinear regression

Table 1. Peak Plasma Levels of Circulating IL-8 After a Single Bolus Injection of IL-8

Monkey	Bodyweight (kg)	Dose of IL-8 (µg/kg)	Plasma Peak Level (ng/mL)
89	2.90	0	8
9141	3.00	10	307
C89	2.90	10	153
9201	2.50	30	482
9123	3.20	30	396
9142	3.90	30	717
9142	3.75	100	610
9137	3.00	100	2,314
9201	2.50	100	8,766
9141	2.90	100	2,510

Peak-levels of circulating IL-8 at 1 minute after injection of rhuIL-8. Monkey identification numbers are presented in the first column.

analysis, using the Biosoft (Cambridge, UK) scientific parameter fit software program P.fit, version 5.1 (Fig.P Software Corp, Durham, NC).

Monoclonal antibody labeling for fluorescence-activated cell sorting (FACS) analysis. Commercially available fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated monoclonal antibodies CD2, CD4, CD8, CD14, CD34, CD20, and CD56 (Becton Dickinson, Mountain View, CA) were used. FN18, an antibody for rhesus CD3 (obtained from Dr M. Jonker, TNO) was indirectly labeled with FITC conjugate goat-antimouse IgG. Data were obtained by sorting at least 10,000 cells using a FACScan flow cytometer (FACScan; Becton Dickinson) with a single argon (488 nm) laser (Becton Dickinson). FITC intensity was measured using a combined 510/515 long-pass and 540 short-pass filter set. PE intensity was measured using a 580/590 long-pass filter.

Experimental protocol. Monkeys were placed in a chair-like restraining device and blood was drawn from the right calf vein (t = 0 sample). Using the same route, saline or IL-8 was injected as a time-controlled bolus injection. Venous blood samples were always obtained from the left calf vein at several time intervals after injection.

Statistical analysis. Differences were evaluated using the Student's *t*-test. *P* values of $<.05$ were considered to be statistically significant.

RESULTS

Pharmacokinetics of IL-8. To study the pharmacokinetics of IL-8, plasma levels of IL-8 were determined at various time intervals after injection. At 1 minute after injection of IL-8, maximum levels of IL-8 were reached ranging from 230 ± 109 ng/mL for animals treated with a dose of 10 μg/kg (mean ± SD, n = 2) up to 3,550 ± 3,580 for animals treated with a dose of 100 μg/kg (mean ± SD, n = 4; Fig 1). No circulating IL-8 could be detected after 120 minutes. Interindividual peak values varied considerably between 610 ng/mL up to 8,766 ng/mL for a dose of 100 μg IL-8 per kilogram of body weight (Table 1). The half-life time for circulating IL-8 calculated from these data was 9.9 ± 2.2 minutes (mean ± SD, n = 12, elimination phase). Within the detection limits, the levels of circulating IL-8 decreased in a monophasic fashion.

Effect of IL-8 on peripheral blood leukocyte counts. All animals injected with IL-8, regardless of the dose, showed an instant neutropenia occurring between 1 and 5 minutes after injection (Fig 2A). The numbers of circulating lymphocytes and monocytes decreased only slightly (Fig 2C). This neutropenia was followed by neutrophilia. At lower doses, maximum levels of circulating neutrophils were reached at earlier time intervals after IL-8 injection (t = 30 for 10 μg/kg, t = 45 for t = 30 μg/kg, and t = 120 for 100 μg/kg; Fig 2A).

The neutrophil fraction consisted of mature and immature cells (Fig 2B). Not only were bands seen, but, in several experiments, myelocytes, metamyelocytes, and erythroblasts were also seen (data not shown). Upon FACS analysis using monoclonal antibodies against CD2, CD3, CD4, CD8, CD20, and CD56, no significant changes between lymphocyte subsets were observed (data not shown).

Distribution of labeled leukocytes after injection of IL-8 as measured by scintigraphy. In 1 of 18 experiments, a

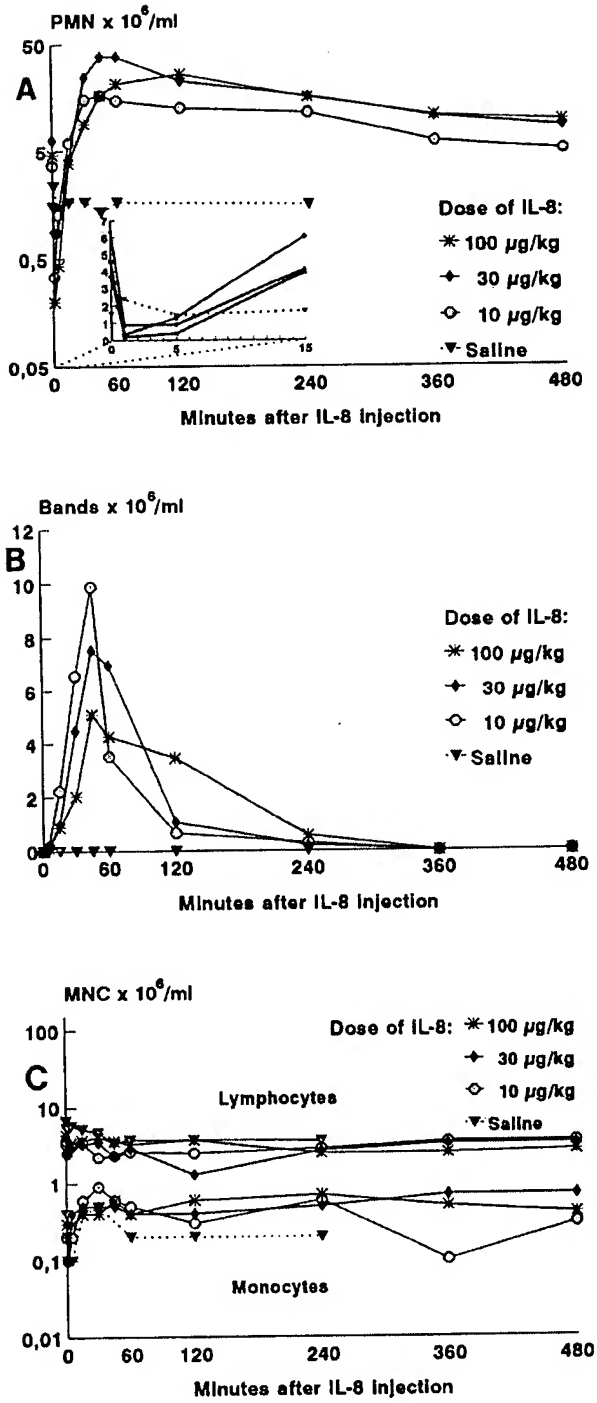


Fig 2. Effect of injection of IL-8 on the numbers of circulating neutrophils (A), bands (B), and mononuclear cells (C). Saline (n = 1), IL-8 (10, 30, or 100 μg/kg) was injected intravenously at t = 0. The inserted figure in (A) is an expanded view of the first 15 minutes after injection of IL-8. Data are expressed as the means of two (10 μg/kg), three (30 μg/kg), or eight (100 μg/kg) experiments.

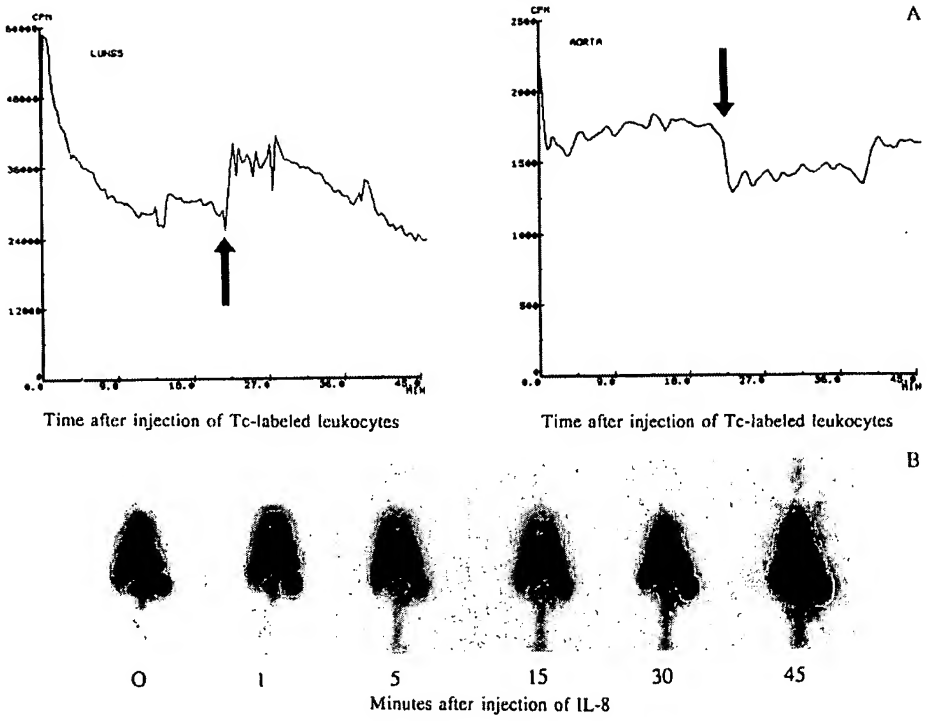


Fig 3. Pulmonary sequestration of leukocytes in the lung after injection of IL-8. Autologous Technetium-labeled leukocytes were reinfused and, after reaching equilibrium, IL-8 (100 μg/kg) was injected at t = 0, as indicated by the arrow (A). Accumulated activity was measured every 30 seconds. An instant decrement of activity above the abdominal aorta coincided with increased activity over the lungs (A). Total body scintigrams show increased activity over the lungs immediately after IL-8 injection (B).

rhesus-monkey became dyspnoeic 1 minute after the injection of IL-8. Dyspnoea resolved spontaneously after 5 minutes. Because this period coincided with the neutropenic phase, we tested the hypothesis that the IL-8–induced neutropenia was caused by sequestration of neutrophils in the lung. A rhesus monkey therefore received an injection of autologous leukocytes labeled with ^{99m}Tc. At 22 minutes after injection, equal distribution of labeled leukocytes over the body had occurred and IL-8 (100 μg/kg) was injected. Immediately after injection and coinciding with the neutropenia, an increment of radioactivity was observed over the lungs concurring with a decrease of activity over the aorta and the brains. No change in radioactivity was observed over the liver or the spleen (Fig 3).

Mobilization of myeloid progenitor cells. To study a possible mobilizing effect, IL-8 was injected intravenously at dose levels of 10, 30, or 100 μg per kilogram of body weight. This resulted in a dose-dependent increase in the numbers of circulating progenitor cells starting at 5 minutes after injection and reaching maximum levels at t = 30 minutes of 1,382 ± 599 CFU/mL for 100 μg IL-8 per kilogram (mean ± SD, n = 8), whereas no increase was observed in control monkeys treated with the vehicle alone (9 ± 7 CFU/mL for saline, mean ± SD, n = 2, t = 30 minutes; Fig 4). The increment in the absolute numbers of progenitor cells ranged from 10 to 100 fold at a dose of 100 μg IL-8 per kilogram. The various types of mobilized progenitor cells were not different between blood derived from IL-8–treated animals and controls (Fig 5).

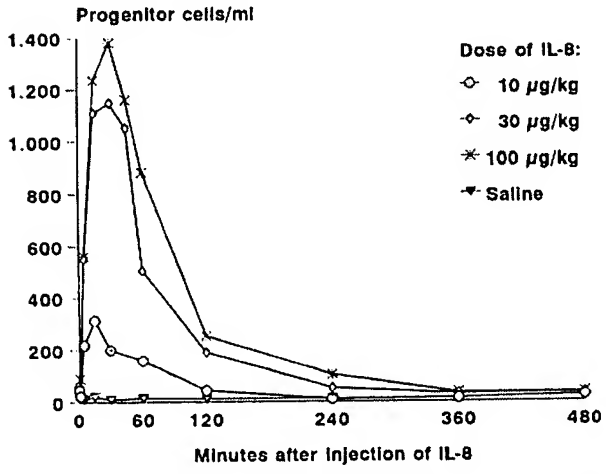


Fig 4. Dose-dependent mobilization of progenitor cells after injection of IL-8 at t = 0. Results are expressed as the means of two (saline and 10 μg/kg), three (30 μg/kg), or eight (100 μg/kg) experiments.

Effect of a second bolus injection of IL-8. To study the possibility of tachyphylaxis, a second bolus injection of IL-8 was administered. An interval of 72 hours was chosen to ensure full restoration of the mobilizing capacity. A second injection resulted in similar levels and kinetics of circulating numbers obtained after the first injection (Fig 6).

DISCUSSION

In this study, we report the stem cell mobilizing properties of IL-8 in rhesus monkeys. Rhesus monkeys treated with a single intravenous bolus injection of IL-8 showed an instant

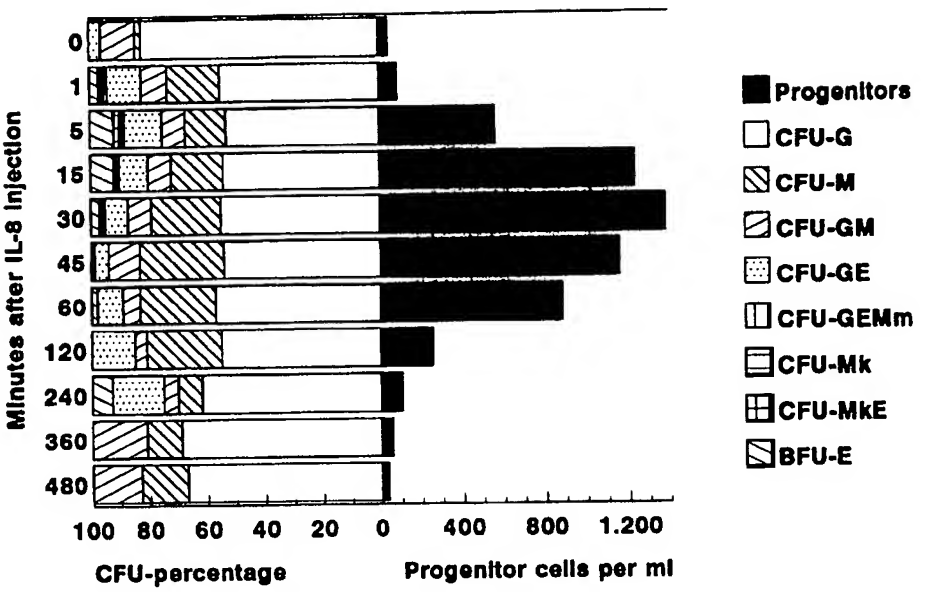


Fig 5. Distribution of progenitor subtypes as determined in a CFU-Mix assay as a percentage (left) of the total numbers of IL-8 (100 μg/kg) mobilized progenitor cells (right). Results are expressed as the means of four experiments. Cells were plated in triplets for each experiment.

high level of circulating IL-8 that rapidly declined. The t of free plasma IL-8 was calculated to be 9.9 ± 2.2 minutes, which is in accordance with the t described by van Zee et al[29] (7.5 ± 1.6 minutes).

IL-8 injection had a profound effect on the numbers of hematopoietic progenitor cells (HPCs) in the peripheral blood. Within 5 minutes after injection, the numbers of circulating HPCs increased rapidly. Maximum levels of circulating progenitor cells were reached at 30 minutes after injection, regardless of the dose. In contrast, the maximum numbers of mobilized progenitor cells were clearly dose-dependent, although considerable interindividual differences were observed, ranging from a 10- to 100-fold increment (mean, 28-fold; n = 8) in circulating HPCs for a dose of 100 μg IL-8 per kilogram of body weight. These interindividual differences could not be explained by differences in levels of circulating IL-8. We previously reported that the mobilization of progenitor cells in mice was a specific effect of IL-8, because the mobilizing and neutrophilia-inducing effect was completely blocked by treatment of the animals with a polyclonal anti-IL-8 antibody before IL-8 injection.[36] In contrast to mature circulating leukocytes, the progenitor cells appearing in the circulation after injection of IL-8 were not lineage-specific. All subtypes of colony-forming cells were observed in the circulation of IL-8-treated animals. Based on the numbers that are commonly used in blood stem cell transplantation in humans (20×10^4 CFU-GM per kilogram of body weight), a leukapheresis procedure processing 300 mL of blood would be sufficient for autologous stem cell transplantation. These data illustrate the potential application of IL-8 in blood stem cell transplantation.

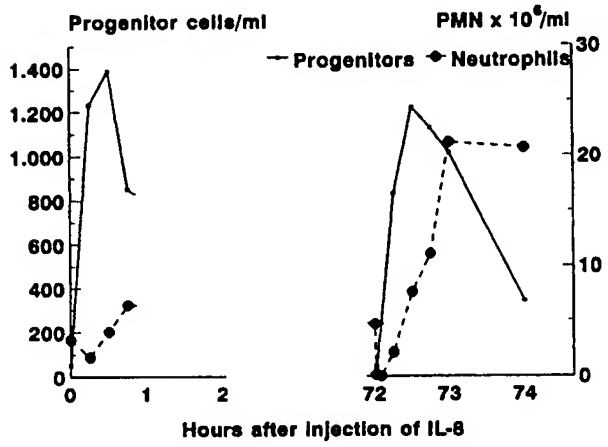


Fig 6. Effect of a second bolus injection of IL-8 on mobilization of progenitor cells and neutrophils (n = 1). A second bolus injection of IL-8 (100 μg/kg) was administered with an interval of 72 hours.

Rapid release of progenitor cells within 30 minutes is, to our knowledge, a unique property of chemoattractant agents. It was previously reported for complement 5a, another chemoattractant.[39,40] However, the mechanism of stem cell mobilization is unclear. Papayannopoulou and Nakamoto[41] have shown that involvement of adhesion molecules plays a role in mobilization of progenitor cells by treating nonhuman primates with injections of anti-VLA_4 antibody. They reported increments in the numbers of HPCs with similar interindividual variety and almost similar levels as reported here. However, mobilization was induced at 24 hours after the first injection of anti-VLA_4 antibody, implicating that another mechanism was involved in IL-8-induced mobilization. Other adhesion molecules such as LFA-1 and L-Selectin are also expressed on hematopoietic progenitor cells.[42-44] IL-8 induces shedding of L-selectin on neutrophils[25,26]; similarly, IL-8 may induce instant shedding of L-selectin of the surface of hematopoietic progenitor cells, contributing to the rapid

mobilization of progenitor cells. However, this hypothesis supposes the expression of IL-8 receptors on HPCs, which is at present unknown.

Another mechanism for rapid mobilization of progenitor cells would be an indirect interference with adhesion of progenitor cells to matrix molecules in the bone marrow microenvironment. Activation of neutrophils by IL-8 induces within a few minutes release of several metalloproteases involved in the degradation of the extracellular matrix.[4,5,20-22] Primitive progenitor cells are preferentially bound to the extracellular matrix molecules.[45] Neutrophils in the bone marrow may release these proteases after activation by IL-8,[46] resulting in nonspecific release of progenitor cells into the circulation. Experiments in primates are in progress to test this hypothesis.

Systemic administration of IL-8 induces an immediate neutropenia also described by others.[29,34-36] The neutropenic period in our experiments lasted for at least 5 minutes. In this study, we showed that ^{99m}Tc-labeled leukocytes accumulated in the lungs instantly after injection of IL-8, coinciding with a decrement of activity over the aorta. Leukocytes did not accumulate in the liver, spleen, or brain. Because only the numbers of circulating neutrophils decreased significantly, it was likely that the accumulation of radioactivity in the lungs was due to neutrophil sequestration. This may also explain the short and self-resolving period of dyspnoea after injection of IL-8 observed in one of the monkeys. Rot[47] and Hechtman et al[35] observed a similar accumulation of neutrophils in the lungs of rabbits. Considering the very rapid induction of neutropenia, an instantly induced effect must play a principal role in this phenomenon. Instant cell stiffening is described on activation of neutrophils.[48] The decreased deformability may then result in accumulation of neutrophils in the microvessels of the lungs.[35,48] Upregulation of adhesion molecules such as CD11b/CD18 β2-integrins may also participate in the accumulation of neutrophils, but does not explain the very rapid neutropenia and the preference for accumulation in the lungs.[27,29,35,47]

At 5 minutes after injection of IL-8, neutrophil counts started to increase. Maximum numbers were reached 30 to 120 minutes after injection, depending on the dose of IL-8 administered. This phenomenon is less well explained. IL-8 promotes neutrophils to adhere to endothelium followed by migration into tissues.[26,49,50] As shown by Hechtman et al,[35] intravascular IL-8 inhibits the migration of neutrophils into tissues. This may be due to the shedding of L-selectin by neutrophils.[25,26] These selectins are necessary for the initial contact of neutrophils before a more potent adhesion via β2-integrins and transendothelial migration.[50] In this way, shedding of L-selectin may also induce circulation of marginating neutrophils. IL-8 is also a chemotactic agent for neutrophils and may recruit neutrophils from the bone marrow reservoir.[4,5,7,37] As shown here, the increment in numbers of neutrophils comprises mature as well as immature cells derived from the bone marrow. This phenomenon was also observed by Hechtman et al.[35] Therefore, recruitment of neutrophils from the marginal pool as well as the bone marrow reservoir may be responsible for the observed neutrophilia. These two IL-8–induced mechanisms of neutrophil mobilization may explain the dose-dependent time interval between injection of IL-8 and maximal numbers of circulating neutrophils. The maximal numbers of circulating immature neutrophils (bone marrow compartment) are identical for all dose levels at 30 minutes. However, neutrophils initially trapped in the lungs are released sooner after injection of lower doses of IL-8 due to a shorter effective plasma level of IL-8. Therefore, desequestration of neutrophils from the lungs would account for the earlier peaking.

In summary, our studies show that a single injection of IL-8 induces instant mobilization of hematopoietic progenitor cells in nonhuman primates in a reproducible fashion. Because IL-8 is a rapid inducer of mobilization of hematopoietic progenitor cells, mobilization may be performed electively. Side effects may be very limited because IL-8 is a cytokine at the end of the acute-phase cascade and has been shown not to induce release of other acute-phase proteins in vivo.[29] Therefore, IL-8 may be potentially applicable for stem cell mobilization in humans.

ACKNOWLEDGMENT

We thank Judy Paauwe of the Laboratory of Clinical Hematology for performing all manual differential counts on blood films. We are also indebted to Noud Neervoort for excellent animal handling and care.

REFERENCES

1. Miller MD, Krangel MS: Biology and biochemistry of the chemokines: A family of chemotactic and inflammatory cytokines. Crit Rev Immunol 12:17, 1992

2. Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K: Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol 9:617, 1991

3. Kownatzki E, Kapp A, Uhrich S: Novel neutrophil chemotactic factor derived from human peripheral blood mononuclear leukocytes. Clin Exp Immunol 64:214, 1986

4. Yoshimura TK, Matsushima S, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ: Purification of a human monocyte-derived neutrophil chemotactic factor that shares sequence homology with host defense cytokines. Proc Natl Acad Sci USA 84:9233, 1987

5. Schröder J-M, Mrowitz U, Morita E, Christophers E: Purification and partial biochemical characterization of a human monocyte-derived neutrophil-activating peptide that lacks interleukin 1 activity. J Immunol 139:3474, 1987

6. Walz A, Peveri P, Aschauer AO, Baggiolini M: Purification and amino acid sequencing of NAF, a novel neutrophil activating factor produced by monocytes. Biochem Biophys Res Commun 149:755, 1987

7. Van Damme J, Van Beeumen J, Opdenakker G, Billiau A: A novel nh_2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive and granulocytosis-promoting activity. J Exp Med 167:1364, 1988

8. Ahuja SK, Özçelik T, Milatovitch A, Francke U, Murphy PM: Molecular Evolution of the human interleukin-8 receptor gene cluster. Nat Genet 2:31, 1992

9. Bazzoni F, Cassatella MA, Rossi F, Ceska M, Dewald B, Baggiolini M: Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin-8. J Exp Med 173:771, 1991

10. Gimbrone MA Jr, Obin MS, Brock AF, Luis EA, Hass PE, Hebert CA, Kip YK, Leung DW, Lowe DG, Kohr WJ, Darbonne WC, Bechtol DB, Baker JB: Endothelial interleukin-8: A novel inhibitor of leukocyte-endothelial interactions. Science 246:1601, 1989

11. Strieter RM, Phan SH, Showell HJ, Remick DG, Lynch JP, Genord M, Raiford C, Eskandri M, Marks RM, Kunkel SL: Monokine-induced neutrophil chemotactic factor gene expression in human fibroblasts. J Biol Chem 264:10621, 1989

12. Standiford TJ, Kunkel SL, Basha MA, Chensue SW, Lynch JP, Toews GB III, Westwick G, Strieter RM: Interleukin-8 gene expression by a pulmonary epithelial cell line: A model for cytokine networks in the lung. J Clin Invest 86:1945, 1990

13. Möller A, Lippert U, Lessmann D, Kolde G, Hamann K, Welker P, Schadendorf D, Rosenbach T, Luger T, Czarnetzki BM: Human mast cells produce IL-8. J Immunol 151:3261, 1993

14. Schröder JM, Mrowietz U, Christophers E: Purification and partial biologic characterization of a human lymphocyte-derived peptide with potent neutrophil-stimulating activity. J Immunol 140:3534, 1988

15. Larsen CG, Anderson AO, Oppenheim JJ, Matsushima K: Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumor necrosis factor. Immunology 68:31, 1989

16. Matsuhima K, Morishita K, Yoshimura T, Lavn S, Kobayashi Y, Lew W, Appella E, Kung HF, Leonard EJ, Oppenheim JJ: Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF MRNA by interleukin-1 and tumor necrosis factor. J Exp Med 167:1883, 1988

17. Strieter RM, Kunkel SL, Showell HJ, Remick DG, Phan SH, Ward PA, Marks RM: Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS and IL-1β. Science 243:1467, 1989

18. Matsushima K, Oppenheim JJ: Interleukin-8 and MCAF: Novel inflammatory cytokines inducible by IL-1 and TNF. Cytokine 1:2, 1989

19. Cluitmans FHM, Esendam BHJ, Landegent JE, Willemze R, Falkenburg JHF: Regulatory effects of T cell lymphokines on cytokine gene expression in monocytes. Lymphokine Cytokine Res 12:457, 1993

20. Lindley I, Aschauer H, Seifert JM, Lam C, Brunowsky W, Kownatzki E, Thelen M, Peveri P, Dewald B, von Tscharner V, Walz A, Baggiolini M: Synthesis and expression in Escherichia coli of the gene encoding monocyte-derived neutrophil-activating factor: Biological equivalence between natural and recombinant neutrophil-activating factor. Proc Natl Acad Sci USA 85:9199, 1988

21. Peveri P, Walz A, Dewald B, Baggiolini M: A novel neutrophil-activating factor produced by human mononuclear phagocytes. J Exp Med 167:1547, 1988

22. Elford PR, Cooper PH: Induction of neutrophil-mediated cartilage degradation by interleukin-8. Arthritis Rheum 34:325, 1991

23. White MV, Yoshimura T, Hook W, Kaliner MA, Leonard EJ: Neutrophil attractant/activation protein-1 (NAP-1) causes human basophil histamine release. Immunol Lett 22:151, 1989

24. Krieger M, Brunner T, Bischoff SC, von Tscharner V, Walz A, Moser B, Baggiolini M, Dahinden CA: Activation of human basophils through the IL-8 receptor. J Immunol 149:2662, 1992

25. Luscinskas FW, Kiely JM, Ding H, Obin MS, Hebert CA, Baker JB, Gimbrone MA: In vitro inhibitory effect of IL-8 and other chemoattractants on neutrophil-endothelial adhesive interactions. J Immunol 149:2163, 1992

26. Smith CW, Kishimoto TK, Abbass O, Hughes B, Rothlein R, McIntire LV, Butcher E, Anderson DC: Chemotactic factors regulate lectin adhesion mole cule 1 (LECAM-1)-dependent neutrophil adhesion to cytokine-stimulated endothelial cells in vitro. J Clin Invest 87:609, 1991

27. Detmers PA, Lo SK, Olsen-Egbert E, Walz A, Baggiolini M, Cohn ZA: Neutrophil-activating protein-1/interleukin-8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. J Exp Med 171:1155, 1990

28. Smith WB, Gamble JR, Clark-Lewis I, Vadas MA: Interleukin-8 induces neutrophil transendothelial migration. Immunology 72:65, 1991

29. Van Zee KJ, Fischer E, Hawes AS, Hebert CA, Terrell TG, Baker JB, Lowry SF, Molldawer LL: Effects of intravenous IL-8 administration in nonhuman primates. J Immunol 148:1746, 1992

30. Colditz IG, Zwahlen RD, Baggiolini M: Neutrophil accumulation and plasma leakage induced in vivo by neutrophil-activating peptide-1. J Leukoc Biol 48:129, 1990

31. Rampart M, Van Damme J, Zonnekeyn L, Herman AG: Granulocyte chemotactic protein/interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. Am J Pathol 135:21, 1989

32. Kunkel SL, Standiford T, Stephens KE, Hatherhill JR, Tazelaar HD, Raffin TA: Interleukin-8 (IL-8): The major neutrophil chemotactic factor in the lung. Exp Lung Res 17:17, 1991

33. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM: Interleukin-8 as a macrophage-derived mediator of angio genesis. Science 258:1798, 1992

34. Jagels MA, Hugli TE: Neutrophil chemotactic factors promote leukocytosis. J Immunol 148:1119, 1992

35. Hechtman DH, Cybulsky MI, Fuchs HJ, Baker JB, Gimbrone MA: Intravascular IL-8: Inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. J Immunol 147:883, 1991

36. Laterveer L, Lindley IJD, Hamilton MS, Willemze R, Fibbe WE: Interleukin-8 induces rapid mobilization of hema topoietic stem cells with radioprotective capacity and long term myelo-lymphoid repopulating ability. Blood 85:2269, 1995

37. Rot A: Chemotactic potency of recombinant human neutrophil attractant/activation protein-1 (interleukin-8) for polymorphonuclear leukocytes of different species. Cytokine 3:21, 1991

38. Zwierzina H, Holzinger I, Gaggi S, Wolf H, Schollenberger S, Lam C, Bammer T, Geissler D, Lindley ID: Recombinant human interleukin-8 restores function in neutrophils from patients with myelodysplastic syndromes without stimulating myeloid progenitor cells. Scand J Immunol 37:322, 1993

39. Benner R, Rijnbeek AM, Molendijk W, Vos O: Genetic control of lipopolysaccharide-induced mobilization of CFUs. Dissociation between early and delayed mobilization of CFUs in complement C5-deficient mice and LPS non-responder mice. Cell Tissue Kinet 14:143, 1981

40. Wilschut IJ, Erkens-Versluis ME, Ploemacher RE, Benner R, Vos O: Studies on the mechanism of hematopoietic stem cell (CFUs) mobilization. A role of the complement system. Cell Tissue Kinet 12:299, 1979

41. Papayannopoulou T, Nakamoto B: Peripheralization of hemopoietic progenitors in primates treated with anti-VLA$_4$ integrin. Proc Natl Acad Sci USA 90:9374, 1993

42. Terstappen LWMM, Huang S, Bui N, Picker LJ: Induction of human hematopoietic stem cell outgrowth by L-Selectin ligation. Blood 82:109a, 1993 (abstr, suppl 1)

43. Liesveld JL, Winslow JM, Frediani KE, Ryan DH, Abboud CN: Expression of integrins and examination of their adhesive function in normal and leukemic hematopoietic cells. Blood 81:112, 1993

44. Watt SM, Williamson J, Genevier H, Fawcett J, Simmons DL, Hatzfeld A, Nesbitt SA, Coombe DR: The heparin binding PECAM-1 adhesion molecule is expressed by CD34$^+$ hematopoietic

precursor cells with early myeloid and B-lymphoid cell phenotypes. Blood 82:2649, 1993

45. Coulombel L, Eaves AC, Eaves CJ: Enzymatic treatment of long-term human-marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. Blood 62:291, 1983

46. Masure S, Nys G, Fiten P, Van Damme J, Opdenakker G: Mouse gelatinase-B: cDNA cloning, regulation of expression and glycosylation on WEHI-3 macrophages and gene organization. Eur J Biochem 218:129, 1993

47. Rot A: Some aspects of NAP-1 pathophysiology: Lung damage caused by a blood-borne cytokine. Adv Exp Med Biol 305:127, 1991

48. Worthen GS, Schwab B III, Elson EL, Downey GP: Mechanics of stimulated neutrophils: Cell stiffening induces retention in capillaries. Science 245:183, 1989

49. Rot A: Endothelial cell binding of NAP-1/IL-8: Role of neutrophil emigration. Immunol Today 13:291, 1992

50. Lawrence MB, Springer TA: Leucocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. Cell 65:859, 1991

CURRICULUM VITAE

HELEN M. BLAU

Donald E. and Delia B. Baxter Professor

Address: Director, Baxter Laboratory in Genetic Pharmacology
Stanford University School of Medicine
269 Campus Drive, CCSR 4215
Stanford, CA 94305-5175
Telephone: (650) 723-6209 Fax: (650) 736-0080
e-mail: hblau@stanford.edu

Personal Data: Birthplace: London, England
Dual Citizenship: USA & Great Britain
Languages: French and German

Education:

1969	B.A.	University of York, York, England
1970	M.A.	Harvard University, Cambridge, MA
1975	Ph.D.	Harvard University, Cambridge, MA

Professional Experience:

1969 - 1975: Predoctoral Fellow, Department of Biology, Harvard University, Cambridge, MA (advisor: F.C. Kafatos)

1975 - 1978: Postdoctoral Fellow, Division of Medical Genetics, Departments of Biochemistry and Biophysics, University of California, San Francisco, CA (advisor: C.J. Epstein)

1978 - 1986: Assistant Professor, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA

1986 - 1991: Associate Professor, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA

1991 - 1999: Professor, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA

1997 - 2002: Chair, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA

1997 - present: Director, Gene Therapy Technology, Stanford University School of Medicine, Stanford, CA

1999 - present: Donald E. and Delia B. Baxter Professor, Stanford University School of Medicine, Stanford, CA

2002 – present: Director, Baxter Laboratory in Genetic Pharmacology, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

2005 – present: Faculty Affiliate, Bio-X Program & Member, Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA

Major Honors and Awards:

Basil O'Connor Faculty Fellow (1978-1981)
Mellon Foundation Faculty Fellow (1979-1980)
William M. Hume Faculty Scholar (1981-1984)
Research Career Development Award, National Institutes of Health (1984-1989)
SmithKline and Beecham Junior Faculty Scholar Award (1989-1991)
Fellow, American Association for the Advancement of Science (AAAS) (1991)
U.S. National Academy of Sciences Delegation to The People's Republic of China (1991)
Resident Scholar, Bellagio Study Center of Rockefeller Foundation, Lake Como, Italy (1992)
Senior Career Recognition Award of WICB, American Society of Cell Biology (1992)
Excellence in Teaching Recognition, Committee on Courses and Curriculum, Stanford University School of Medicine (1992-1993); (1993-1994); (1995-1996); (1997-1998)
54th President, American Society for Developmental Biology (1994-1995)
MERIT Award, National Institutes of Health (1995-2005)
Nobel Forum Lecture, Karolinska Inst., Sweden (1995)
Yvette Mayent-Rothschild Visiting Professor, Instituts Curie and Pasteur, Paris (5 months,1995)
Institute of Medicine of the National Academy of Sciences (elected 1995)
National Advisory Council, NIH Institute on Aging (1996-2000)
American Academy of Arts and Sciences (elected 1996)
Initiator and Liaison, Stanford/Aventis Inc. $5 Million Innovative Grant Agreement (1997-2002)
NATO Collaborative Research Programme, France (1997-98)
Board of Directors, American Society for Gene Therapy (1999-2002)
FASEB Excellence in Science Award (1999)
Donald E. and Delia B. Baxter Endowed Professorship (1999)
NIH Director's (Harold Varmus) Lectureship (2000)
President, International Society of Differentiation (2002-2004)
Initiator and P.I. of Aventis/Gencell-Stanford Collaborative Agreement on Angiogenesis for $12 million (2001-2005)
Fellow, American Heart Association, Council on Basic Cardiovascular Sciences (2001)
McKnight Endowment Fund for Neuroscience Award (2001)
Council Member, American Society for Cell Biology (ASCB)(2002-2004)
Honorary Doctorate, University of Nijmegen, Holland (2003)
Rolf-Sammet-Fonds Visiting Professorship, University of Frankfurt (2003)
400th Annual Pontifical Academy, the Vatican in Rome, plenary talk on stem cells, Modern Biotechnologies Symposium, Audience with Pope John Paul II (2003)
Institute of Medicine (IOM):
The Membership Nominating Committee (Chair of Section 2) (2003)

Council Nominating Committee Working Group on Reassessing the IOM sections and elections (2003)
Council Executive Committee Member (2004-2009)
Board Member, Harvard Board of Overseer's (2004-2010)
Founding Board Member, The Rosalind Franklin Society (2007)
Scientific Advisory Board Member, Ellison Medical Foundation (2007-2008)
Fulbright Senior Specialist at the Institut Pasteur, Paris (2007)

Professional Societies:

American Association for the Advancement of Science
American Heart Association
American Society for Biochemistry and Molecular Biology
American Society for Cell Biology
American Society for Developmental Biology
American Society of Gene Therapy
American Society of Human Genetics
Hastings Institute of Society, Ethics, and the Life Sciences
International Society of Differentiation
Society for Neuroscience

Editorial Boards:

The Journal of Cell Biology (1987-1994)
Experimental Cell Research (1987-2000)
Neuromuscular Disorders (1990-1994)
Molecular and Cellular Biology (1991-1994)
Developmental Biology, consulting editor (1995-1998)
Journal of Molecular Medicine (1994-2001)
Advisory Board, Biochimica et Biophysica Acta Reviews on Cancer (1995-2002)
Molecular Therapy (2000-2004)
Molecular Interventions (2000-2006)
Somatic Cell and Molecular Genetics (1988-2002)
Molecular and Cellular Differentiation (1992-present)
Genes to Cells (Senior Editor, 1994-present)
Molecular Medicine Today/Trends in Molecular Medicine (1994-present)
FASEB Journal (Associate Editor, 2005- present)
Differentiation (Senior Editor, 2005-present)

Organizer, National and International Meetings:

Co-organizer with M. Karin, FASEB Meeting, *Regulation of Tissue-Specific Gene Expression* (1990)
Co-organizer with A. Kelly, Keystone Symposium, *Gene Expression in Neuromuscular Development* (1991)
President/Co-organizer with N. Rosenthal, 54th Annual Meeting of the American Society for Developmental Biology, *Genes, Development and Cancer* (1995)

Co-organizer with J. Wilson, Keystone Symposium, *Molecular and Cellular Biology of Gene Therapy* (1997)
Organizer, First Gene Therapy Symposium at Stanford, *Gene Therapy: Prospects for the Next Decade* (1999)
International Scientific Advisory Committee, 8th Meeting of the European Society of Gene Therapy (ESGT), Stockholm, Sweden, Gene Therapy (2000)
Co-organizer with W. Mobley and A. Garber, First Interdisciplinary Aging Symposium at Stanford, *Aging: Biology, Disease and Economics* (2001)
President/Organizer, 13th *International Society of Differentiation Meeting*, Hawaii (2004)
Co-organizer with Z. Hall and C. Laurencian, 2006 Annual Meeting of the Institute of Medicine, *Stem Cells and Tissue Engineering* (2006)

Experience In Training (Students, Postdoctoral Fellows and Medical Residents):

Faculty Affiliate and Preceptor, NIH Cancer Biology Predoctoral/Postdoctoral Training Program, Stanford School of Medicine (1978-present)
Admissions Coordinator, NIH Pharmacology Predoctoral Training Program, Stanford School of Medicine (1981-1988; 1994-1996)
Program Committee, NIH Neurosciences Predoctoral Training Program at Stanford School of Medicine (1987-1991; 1993-1996); Faculty Preceptor (1998-present)
Associate Program Director, NIH Developmental & Neonatal Biology Predoctoral and Postdoctoral Training Program at Stanford School of Medicine (1988-present)
Co-director, CAM with J. Boothroyd (First Stanford Predoctoral Multidepartmental Combined Admission Mode) (1990-1993)
Faculty Affiliate and Preceptor, NIH Biotechnology Predoctoral Training Program, Stanford School of Medicine (1993-present)
Special Advisor, Cell Sciences Program, Stanford School of Medicine (1994-present)
Program Committee, Flexible Graduate Admissions Program at Stanford (1995-1996)
Head, Task Force on Training, National Institute on Aging, Washington, D.C. (1998-1999)
P.I. and Director, NIH Pharmacology Predoctoral Training Program, Stanford School of Medicine (1998-2003)
P.I. and Director, NIH Molecular Pharmacology of Diseases of Aging Predoctoral Training Program, Stanford School of Medicine (1998-2004)
Member, Women's Reproductive Health Research (WRHR) Career Development Program at Stanford School of Medicine (2000-present)
Member, Microbiology and Immunology Department Graduate Steering Committee (2002-2003)
Member, Institute for Stem Cell Biology and Regenerative Medicine, Stanford School of Medicine (2005-present)
Member, Stanford University School of Medicine Advanced Residency Training at Stanford Committee (2007)
Head, CIRM Scholar Training Program, Institute for Stem Cell Biology and Regenerative Medicine (2007-present)
P.I. and Co-Director (with Stuart Kim), NIH Aging and Regenerative Medicine Pre- and Postdoctoral Training Program (2008 start pending)

Selected Administrative Experience (University and Public Service):

Genetic Counseling, Department of Medical Genetics, U.C., San Francisco (1975-1978)
Reproductive Outcome Research for Medfly Health Advisory Committee, State of California (1981-1982)

Faculty Senator for Dept. of Pharmacology, Academic Senate of Stanford School of Medicine (1982-85)
Faculty Affiliate, Institute for Research on Women and Gender, Stanford University (1982-present)
Dean Search Committee, Stanford School of Medicine (1984)
Nominating Committee, American Society for Cell Biology (1985-1986)
Faculty Senator-At-Large, Stanford School of Medicine (1986-1989)
NIH Molecular Cytology Study Section (Ad Hoc) (1987, 1988)
Steering Committee of the Faculty Senate, "Committee of Five," Stanford School of Medicine (1986-1989)
Chair, Dean's Advisory Committee on Junior Faculty Awards, Stanford School of Medicine (1987-1989)
Advisory Board, Program in Molecular and Genetic Medicine at Stanford (1988-1995)
Chair, Katharine Dexter McCormick Distinguished Lectureship Committee, Stanford (1989-1998)
NICHHD/NIH Five-Year Planning Committee, Genetics & Teratology Branch Washington, D.C. (1989)
Program Committee, American Society for Cell Biology (1990, 2004)
Executive Committee, Basic Science Council, American Heart Association (1991-1992)
NIH (RAC) Oversight Committee of Gene Therapy (created by Harold Varmus) (1995)
Chair, Nominating Committee, American Society for Biochemistry and Molecular Biology (1996)
Co-Chair, Dean's Task Force on Gene Therapy, Stanford Medical School (1995-1996)
National Advisory Council, National Institute on Aging, NIH, Washington, D.C. (1996-2000)
Board of Directors, American Society of Gene Therapy (1998-2001)
Director, Gene Therapy Technology, Stanford School of Medicine (1998-present)
Co-Organizer (with Mark Kay), Frontiers in Cell and Gene Therapy Seminar Series, Stanford (1999-present)
Co-Organizer, French Rhône-Poulenc Rorer/Stanford Workshop on Gene Therapy (1999)
American Society for Cell Biology (ASCB), Joint Steering Committee on Public Policy (1999-present)
Member, Congressional Liaison Committee for Public Policy for ASCB (2000-present)
Chair, American Society of Gene Therapy, Scientific Committee for 3rd Annual Meeting on Neuromuscular Disorders (2000); Committee Member (2003)
Fellow, American Heart Association, Council on Basic Cardiovascular Sciences (2001-present)
Chair, Membership Committee, Institute of Medicine (IOM) of the National Academy of Sciences Membership Committee, Section 02 (2002-2003); Vice Chair (2000-2001)
Membership Committee, American Academy of Arts and Sciences, Class II Biological Sciences, Section 2 (2001, 2002)
Scientific Planning Committee Member, Conseil Stratégique de l'Association Française contre les Myopathies (AFM) (2001-2003)
Scientific Coordinator, Stanford Interdisciplinary Institute for French Studies (2002-present)
Member, International Society for Stem Cell Research Education Subcommittee (2003-present)
Faculty Affiliate, Stanford Cardiovascular Institute (2003-present)
Member, International Society for Stem Cell Research Education Subcommittee (2003-2004)
Member, Institute of Medicine Nominating Committee (2004)
Member, IOM Council Nominating Committee (2005)
Member, IOM Working Group on Physical and Basic Biomedical Sciences, Reorganization of sections (2004)
Faculty Affiliate, Stanford Center for Longevity (2004-present)

Faculty Affiliate, Stanford Bio-X Program (2005-present)
Member, Education Committee, Stanford Program in Regenerative Medicine (2005-present)

Selected Honorary Lectureship Invitations (last 5 years):

Plenary Session Chair and Speaker, Gene Therapy 2001: A Gene Odyssey, Keystone Symposium, Utah (2001)
Co-Chair and Speaker, Stem Cell Minisymposium, 41st Meeting of the American Society for Cell; Biology (ASCB), Washington, DC (2001)
Broadhurst Distinguished Lecturer, Harvard University, Cambridge, MA (2001)
Keynote Speaker, SBF 488 Symposium: Neural and Non-Neural Stem Cells, Heidelberg, Germany (2001)
Plenary Speaker, Gordon Research Conference on Angiogenesis and Microcirculation, Salve Regina University, Newport, RI (2001)
Plenary Speaker, Myogenesis, Gordon Research Conference, Il Ciocco, Italy (2001); Discussion Leader and Plenary Speaker (2004)
Mayer Lecture in the Life Sciences, Massachusetts Institute of Technology, Cambridge, MA (2002)
Mary Elizabeth Garrett Lecturer, Johns Hopkins University School of Medicine, Baltimore, MD (2002)
Catherine N. Stratton Lecturer on Critical Issues, Whitehead Institute, Massachusetts Institute of Technology, MA (2002)
President's (Harold Varmus) Research Seminar Series Lecturer, Memorial Sloan Kettering Cancer Center, NY (2002)
Plenary Speaker, British Society for Developmental Biology, Spring Meeting, University of Warwick, England (2003)
Plenary Speaker, Perlmutter Family Symposium on Neurodegenerative Disorders, Harvard University School of Medicine (2003)
2003 Annual Scientist in Medicine Lecture, University of Washington (2003)
Rolf-Sammet-Fonds Visiting Professorship, University of Frankfurt (2003)
Honorary Doctorate, University of Nijmegen, Holland (2003)
Speaker, Academie des Sciences de la France at Pontifical Academy, the Vatican in Rome, "Stem Cells and Cloning" (2003)
Plenary Speaker, XIIIth International Vascular Biology Meeting, Toronto, Canada (2004)
Plenary Speaker, 2nd Annual International Society for Stem Cell Research Meeting, Boston, MA (2004)
President's Lecture, 13th International Society of Differentiation Conference, Honolulu, HI (2004)
Keynote Speaker, Allergan Foundation Lectures in Modern Biology and the Howard A. Schneiderman Memorial Bioethics Lecture Series, University of California, Irvine (2004)
Louis A. Bloomfield Lecturer, Case Western Research University School of Medicine, Cleveland, OH (2005)
Speaker, Inaugural Gurdon Institute Symposium, Wellcome Trust / Cancer Research UK Gurdon Institute, Cambridge, UK (2005)
Plenary Speaker and Session Chair, Summer Research Conference: Skeletal Muscle Satellite and Stem Cells, FASEB, Tucson, AZ (2005)
Speaker, Senior Ellison Grantee, Colloquium on the Biology of Aging, Ellison Medical Foundation, Woods Hole, MA (2005)

Symposium Chair and Plenary Speaker, Heinz Herrmann Symposium: Reprogramming Cell Fate, American Society for Cell Biology, San Francisco, CA (2005)
Stem Cells and Aging Meeting, National Institute on Aging, Potomac, MD (2006)
Frontiers in Myogenesis, Society for Muscle Biology, Pine Mountain, GA (2006)
Stem Cells and Cancer, German Cancer Research Center, Heidelberg, Germany (2006)
Plenary Session Chair and Speaker, Embryonic and Somatic Stem Cells, German Research Foundation and the German Academy of Sciences Leopoldina, Dresden, Germany (2006)
Opening Lecturer, EMBO Conference on the Molecular and Cellular Basis of Regeneration and Tissue Repair, Ascona, Switzerland (2006)
Keynote Speaker on Stem Cells for Brain in annual meeting: The Role of Stress in Psychiatric and Medical Disorders, The American College of Psychiatrists, San Juan, Puerto Rico (2006)
Friday Evening Lecturer, Colloquium on the Biology of Aging, Woods Hole, MA (2006)
Invited Lecturer, Regenerative Cell Therapy, International Symposium of the French Academy of Sciences, Paris, France (2006)
Plenary Session Chair and Speaker, Stem Cell Interactions with their Microenvironmental Niche, Keystone Symposium, Colorado (2007)
Plenary Speaker, Gordon Research Conference on Myogenesis Conference, Il Ciocco, Italy (2007)
Invited Speaker, Swiss Institute for Experimental Cancer Research in Lausanne, Switzerland (2007)
Invited Speaker, Friedrich Miescher Institut Student Seminar in Basel, Switzerland (2007)

ONGOING RESEARCH

Formatted

- **Stem Cell Biology/Cell Differentiation**: We are elucidating the molecular mechanisms underlying nuclear reprogramming critical to adult stem cell function in skeletal muscle and brain and testing their application to mouse models of disease.

- **Bioengineered Niches:** We are producing nanochips to study the role of extrinsic factors in cell fate determination and self-renewal of single stem cells. The effects on apoptosis, cell division, and differentiation of soluble components (growth factors and cytokines) and tethered insoluble components (cell-cell adhesion and extracellular matrix) are being monitored in hydrogel microwells by time lapse microscopy.

- **Elucidation of signal transduction pathways**: Using technologies developed in our laboratory (restriction enzyme generated siRNAs (REGS) for loss of function analyses and β-galactosidase assays of protein complementation for monitoring intracellular protein translocation, we are determining the molecular bases (chromatin remodeling and signaling pathways) for changing the nuclear function of embryonic and adult stem cells.

Current Active Grants:

Source (all as P.I.)	Title	Total Period
NIH AG009521 (R37) (MERIT – 21 years)	Activators of Human Muscle Genes	4/1/86-3/31/2010

NIH HD018179 (R01) (24 years)	Regulation of Muscle Development	7/1/83-12/31/2007
NIH AG020961 (R01)	Stem Cells for Brain and Brawn Competitive renewal under "Regulation of Muscle Stem Cell Fate" pending	5/1/02-4/30/2007
NIH AG024987 (R01)	Regulators of Adult Stem Cell Fate	10/1/04-9/30/2009
NIH R01 EB005011 (PI M. Bogyo)	Small Molecular Imaging of Cysteine Protease Activity	12/01/05-11/30/10
Stanford Bio-X IIP3-34	Determination of Muscle Stem Cells in Bioengineered Artificial Niches	10/01/06-09/30/08

Patents:

(1) 1989: Isolation, growth, differentiation and genetic engineering of human muscle cells, Patent No. 5,538,722 – Stanford Docket S89-054 (issued 7/23/96).

(2) 1997: Detection of molecular interactions by reporter subunit complementation, U.S. Patent No. 6,342,345 B1 - International Application No. PCT/US98/06648 – Stanford Docket S96-125 (issued 1/29/02).

(3) 1997: RetroTet ART: Retroviral Tetracycline regulatable vector system in which Activators of Repressors are expressed Together (pending).

(4) 2002: Detection of Molecular Interactions by Beta-lactamase Reporter Fragment Complementation (pending)

(5) 2003: Methods and compositions for use in preparing shRNAs (pending).

(6) 2003: Biological Sensors for Protein Interactions: Detection of Protein Translocation by β-Galactosidase Reporter Fragment Complementation (pending)

(7) 2004: Methods for treating disorders of neuronal deficiency with bone marrow-derived cells (pending).

(8) 2005: *In Vivo* Luminescent Detection of beta-Galactosidase Activity (pending)

(9) 2006: GPCR Functional Assay (pending)

(10) 2007: Detection of Molecular Interactions Using a Reduced Affinity Enzyme Complementation Reporter System (pending)

Training Grants (Pending, Active and Former):

NIA T32 (Pending)	Aging and Regenerative Medicine	7/1/08-6/31/13

(P.I.: H. M. Blau, Co-Director: S. Kim)	Training Grant	
NICHHD HD07249 (T32) (P.I.: D.K. Stevenson, Assoc. Program Director: H.M. Blau)	Developmental and Neonatal Biology Training Grant	5/1/04-4/30/2009
NIA AG00259 (T32) (P.I.: H. M. Blau)	Molecular Pharmacology of Diseases of Aging Predoctoral Training Grant	9/1/98-8/31/2004
NIGMS GM07149 (T32) (P.I.: H. M. Blau)	Predoctoral Pharmacology Training Grant	7/1/98-6/30/2003

Teaching:

1969-74:	Teaching Fellow, Harvard University: courses in Introductory Biology, Cell Biology, and Human Genetics
1972-74:	Resident Tutor in Biology, Adams House, Harvard University
1973-74:	Human Genetics: Undergraduate level seminar course taught by HMB at Harvard University
1980 Spring:	Pharmacology 203: Pharmacology for Medical Students, Course coordinator, Stanford University
1981 Winter:	Pharmacology 209: Developmental Biology: Genetic and Environmental Influences, Stanford University (graduate level course directed by HMB)
1984, 87, 90:	Pharmacology 231: Regulation of Gene Expression During Differentiation and Development, Stanford University (graduate level course directed by HMB)
1994, 95, Spring:	Pharmacology 240: Course and Seminar Series on "Drug Discovery", Stanford University (graduate level course, lecture by HMB on gene therapy)
1998, 2000-01, Spring:	Pharmacology 231: Course and Seminar Series on "Stem Cells and Gene Therapy", Stanford University (graduate level course co-directed by HMB and Garry Nolan)
2000, Spring	Pharmacology 241: Course and Seminar Series on "Gene Therapy for Diseases of Aging" (graduate level course co-directed by HMB, Ellen Porzig and Garry Nolan)

1980-present:	Guest lecturer at Stanford University in *Department of Biology*: in Regulatory Biochemistry in Higher Eukaryotes (248) and in Organismal Development (108); in *Department of Genetics*: in Human Genetics (202) and Somatic Cell Genetics (212); in *Department of Cell Biology*: in Cell Motility (225); in *Department of Molecular and Cellular Physiology*: in Pathophysiology (206); in *Cancer Biology Program*: in Signal Transduction Pathways in Development and Cancer (251) and Gene Regulation and Cell Differentiation (252); and in the *Human Biology Program*: in Human Biology
1978-2001:	Pharmacology 201, 202: Pharmacology for Medical Students, Stanford University (taught by entire Pharmacology Department faculty; lectures by HMB on anticancer agents, anticoagulants, pharmacogenetics, teratogenesis, gout, chemical contraception, and gene therapy)
2001, Winter:	Bio 2: Sophomore Lecture Series (faculty members lecture to undergraduates to inform them about careers in sciences and areas of research)
2002-03, Spring:	Microbiology & Immunology 231: Course and Seminar Series on "Stem Cells and Gene Therapy," Stanford University (graduate level course co-directed by HMB and Garry Nolan)
2004, Winter	Microbiology & Immunology 210: Pathogenesis of Bacteria, Viruses and Eukaryotic Parasites (taught by Microbiology and Immunology Department faculty; lecture by HMB on retroviruses in gene therapy)
2005-2007:	REMS (Regenerative Medicine at Stanford) Seminar Series: interdisciplinary seminar and discussion course to create a forum for Stanford students and researchers to learn about what is going on in regenerative medicine at Stanford. Speakers alternate between outside experts in the field of regenerative medicine and stem cell biology and professors and researchers at Stanford

Publications:

Papers published in refereed scientific journals

1. Blau, H.M. and Kafatos, F.C. (1978) Secretory kinetics in the follicular cells of silkmoths during eggshell formation. J. Cell Biol. 78:131-151.

2. Blau, H.M. and Kafatos, F.C. (1979) Morphogenesis of the silkmoth chorion: Patterns of distribution and insolubilization of the structural proteins. Devel. Biol. 72:211-225.

3. Blau, H.M. and Epstein, C.J. (1979) Manipulation of myogenesis *in vitro*: Reversible inhibition by DMSO. Cell 17:95-108.

4. Blau, H. M. and Webster, C. (1981) Isolation and characterization of human muscle cells. Proc. Natl. Acad. Sci., USA 78:5623-5627.

5. Blau, H.M., Kaplan, I., Tao, T.W. and Kriss, J.P. (1983) Thyroglobulin-independent, cell-mediated cytotoxicity of human eye muscle cells in tissue culture by lymphocytes of a patient with Graves' Ophthalmopathy. Life Sci. 32:45-53.

6. Blau, H.M., Webster, C., Chiu, C.-P., Guttman, S. and Chandler, F. (1983) Differentiation properties of pure populations of human dystrophic muscle cells. Exp. Cell Res. 144:495-503.

7. Gunning, P., Ponte, P, Okayama, H., Engel, J., Blau, H.M. and Kedes, L. (1983) Isolation and characterization of full-length cDNA clones for human alpha-, beta-, and gamma-actin mRNAs: Skeletal but not cytoplasmic actins have an amino terminal cysteine that is subsequently removed. Mol. Cell. Biol. 3:787-795.

8. Blau, H.M., Chiu, C.-P. and Webster, C. (1983) Cytoplasmic activation of human nuclear genes in stable heterokaryons. Cell 32:1171-1180.

9. Blau, H.M., Webster, C. and Pavlath, G.K. (1983) Defective myoblasts identified in Duchenne muscular dystrophy. Proc. Natl. Acad. Sci., USA 80:4856-4860.

10. Ponte, P., Gunning, P., Blau, H.M. and Kedes, L.(1983) Human actin genes are single copy for α-skeletal and α-cardiac actin but multicopy for ß- and γ-cytoskeletal genes: 3' untranslated regions are isotype specific, but are conserved in evolution. Mol. Cell. Biol. 3:1783-1791.

11. Gunning, P., Ponte, P., Blau, H.M. and Kedes, L. (1983) Alpha-skeletal and alpha-cardiac actin genes are co-expressed in adult human skeletal muscle and heart. Mol. Cell. Biol. 3:1985-1995.

12. Chiu, C.P. and Blau, H.M. (1984) Reprogramming cell differentiation in the absence of DNA synthesis. Cell 37:879-887.

13. Bains, W., Ponte, P., Blau, H. and Kedes, L. (1984) Cardiac actin is the major actin gene product in skeletal muscle cell differentiation *in vitro*. Mol. Cell. Biol. 4:1449-1453.

14. Chiu, C.P. and Blau, H.M. (1985) 5-Azacytidine permits gene activation in a previously non-inducible cell type. Cell 40:417-424.

15. Blau, H.M., Pavlath, G.K., Hardeman, E.C., Chiu, C.-P., Silberstein, L., Webster, S.G., Miller, S.C. and Webster, C. (1985) Plasticity of the differentiated state. Science 230:758-766.

16. Pavlath, G.K. and Blau, H.M. (1986) Expression of muscle genes in heterokaryons depends on gene dosage. J. Cell Biol. 102:124-130.

17. Minty, A.J., Blau, H.M. and Kedes, L. (1986) Two-level regulation of cardiac actin gene transcription: Muscle-specific modulating factors can accumulate before gene activation. Mol. Cell. Biol. 6:2137-2148.

18. Webster, C., Filippi, G., Rinaldi, A., Mastropaolo, C., Tondi, M., Siniscalco, M. and Blau, H.M. (1986) The myoblast defect identified in Duchenne muscular dystrophy is not

a primary expression of the DMD mutation: Clonal analysis of myoblasts from double heterozygotes for two X-linked loci: DMD and G6PD. Human Genetics 74:74-80.

19. Kaplan, I.D. and Blau, H.M. (1986) Metabolic properties of human acetylcholine receptors can be characterized on cultured human muscle. Exp. Cell Res. 166:379-390.

20. Costa, E.M., Blau, H.M. and Feldman, D. (1986) 1,25 dihydroxyvitamin D_3 receptors and hormonal responses in cloned human skeletal muscle cells. Endocrinology 119:2214-2220.

21. Silberstein, L., Webster, S.G., Travis, M. and Blau, H.M. (1986) Developmental progression of myosin gene expression in cultured muscle cells. Cell 46:1075-1081.

22. Shimizu, M., Webster, C., Morgan, D.O., Blau, H.M. and Roth, R.A. (1986) Insulin and insulin-like growth factor receptors and responses in cultured human muscle cells. Amer. J. Physiol. 251:E611-E615.

23. Hardeman, E., Chiu, C.-P., Minty, A. and Blau, H.M. (1986) The pattern of actin expression in human fibroblast x mouse muscle heterokaryons suggests that human muscle regulatory factors are produced. Cell 47:123-130.

24. Gunning, P., Hardeman, E., Wade, R., Ponte, P., Bains, W., Blau, H.M. and Kedes, L.(1987) Differential patterns of transcript accumulation during human myogenesis. Mol. Cell. Biol. 7:4100-4114.

25. Soileau, L.C., Silberstein, L., Blau, H.M. and Thompson, W.J. (1987) Reinnervation of muscle fiber types in the newborn rat soleus. J. Neurosci. 7:4176-4194.

26. Webster, C., Pavlath, G.K., Parks, D.R., Walsh, F.S. and Blau, H.M. (1988) Isolation of human myoblasts with the fluorescence-activated cell sorter. Exp. Cell Res. 174:252-265.

27. Miller, S.C., Pavlath, G.K., Blakely, B.T. and Blau, H.M. (1988) Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons. Genes Dev. 2:330-340.

28. Webster, C., Silberstein, L., Hays, A.P. and Blau, H.M. (1988) Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. Cell 52:503-513.

29. Hardeman, E.C., Minty, A., Benton-Vosman, P., Kedes, L. and Blau, H.M. (1988) *In vivo* system for characterizing clonal variation and tissue-specific gene regulatory factors based on function. J. Cell Biol. 106:1027-1034.

30. Blau, H.M. (1988) Hierarchies of regulatory genes may specify mammalian development. Cell 53:673-674.

31. Miller, S.C., Ito, H., Blau, H.M. and Torti, F.M. (1988) Tumor necrosis factor inhibits human myogenesis *in vitro*. Mol. Cell. Biol. 8:2295-2301.

32. Ham, R.G., St. Clair, J.A., Webster, C. and Blau, H.M. (1988) Improved media for normal human muscle satellite cells: Serum-free clonal growth and enhanced growth with low serum. *In Vitro* Cell Dev. Biol. 24:833-844.

33. Blau, H.M. (1989) How fixed is the differentiated state? Lessons from heterokaryons. Trends in Genetics 5:268-272.

34. Pavlath, G.K., Rich, K., Webster, S.G. and Blau, H.M. (1989) Localization of muscle gene products in nuclear domains. Nature 337:570-573.

35. Pavlath, G.K., Chiu, C.-P. and Blau, H.M. (1989) *In vivo* aging of human fibroblasts does not alter nuclear plasticity in heterokaryons. Som. Cell Mol. Genet. 15:191-202.

36. Karsch-Mizrachi, I., Travis, M., Blau, H.M. and Leinwand, L.A. (1989) Expression and DNA sequence analysis of a human embryonic skeletal muscle myosin heavy chain gene. Nucleic Acids Res. 17:6167-6179.

37. Schäfer, B.W., Blakely, B.T., Darlington, G.J. and Blau, H.M. (1990) Effect of cell history on response to helix-loop-helix family of myogenic regulators. Nature 344:454-458.

38. Kaplan, I., Blakely, B.T., Pavlath, G.K., Travis, M. and Blau, H.M. (1990) Steroids induce acetylcholine receptors on cultured human muscle: Implications for myasthenia gravis. Proc. Natl. Acad. Sci., USA 87:8100-8104.

39. Condon, K., Silberstein, L., Blau, H.M. and Thompson, W.J. (1990) Development of muscle fiber types in the prenatal rat hindlimb. Devel. Biol. 138:256-274.

40. Condon, K., Silberstein, L., Blau, H.M. and Thompson, W.J. (1990) Differentiation of fiber types in aneural musculature of the prenatal rat hindlimb. Devel. Biol. 138:275-295.

41. Hughes, S.M. and Blau, H.M. (1990) Migration of myoblasts across basal lamina during skeletal muscle development. Nature 345:350-353.

42. Peterson, C.A., Gordon, H., Hall, Z.W., Paterson, B.M. and Blau, H.M. (1990) Negative control of the helix-loop-helix family of myogenic regulators in the NFB mutant. Cell 62:493-502.

43. Hughes, S.M. and Blau, H.M. (1990) Cell lineage in vertebrate development. Curr. Opin. in Cell Biol. 2:981-985.

44. Webster, C. and Blau, H.M. (1990) Accelerated age-related decline in replicative lifespan of Duchenne muscular dystrophy myoblasts: Implications for cell and gene therapy. Som. Cell Mol. Genet. 16:557-565.

45. Mantegazza, R., Hughes, S.M., Mitchell, D., Travis, M., Blau, H.M, and Steinman, L. (1991) Modulation of MHC class II antigen expression in human myoblasts after treatment with IFN-γ. Neurology 41:1128-1132.

46. Blau, H.M. and Baltimore, D. (1991) Differentiation requires continuous regulation. J. Cell Biol. 112:781-783.

47. Dhawan, J., Pan, L.C., Pavlath, G.K., Travis, M.A., Lanctot, A.M. and Blau, H.M. (1991) Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. Science 254:1509-1512.

48. Hughes, S.M. and Blau, H.M. (1992) Muscle fiber pattern is independent of cell lineage in postnatal rodent development. Cell 68:659-671.

49. Pan, L.C., Margolskee, R.F. and Blau, H.M. (1992) Cloning muscle isoforms of neural cell adhesion molecule using an episomal shuttle vector. Som. Cell Mol. Genet. 18:163-177.

50. Peterson, C.A., Cho, M., Rastinejad, F.,and Blau, H.M. (1992) ß-Enolase is a marker of human myoblast heterogeneity prior to differentiation. Devel. Biol. 151:626-629.

51. Gussoni, E., Pavlath, G.K., Lanctot, A.M., Sharma, K., Miller, R.G., Steinman, L. and Blau, H.M. (1992) Normal dystrophin transcripts detected in DMD patients after myoblast transplantation. Nature 356:435-438.

52. Blau, H.M. (1992) How cells know their place. Nature News & Views 358:284-285.

53. Blau, H.M. (1992) Differentiation requires continuous active control. Annual Review of Biochemistry 61:1213-1230.

54. Rastinejad, F. and Blau, H.M. (1993) Genetic complementation reveals novel regulatory role for 3' untranslated regions in growth and differentiation. Cell 72:903-917.

55. Cho, M., Webster, S.G. and Blau, H.M. (1993) Evidence for myoblast-extrinsic regulation of slow myosin heavy chain expression during muscle fiber formation in embryonic development. J. Cell Biol. 121:795-810.

56. Blau, H.M., Dhawan, J. and Pavlath, G.K. (1993) Myoblasts in pattern formation and gene therapy. Trends In Genetics 9:269-274.

57. Hughes, S.M., Cho, M, Karsch-Mizrachi, I., Travis, M., Silberstein, L., Leinwand, L.A. and Blau, H.M. (1993) Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. Devel. Biol. 158:183-199.

58. Blau, H.M. (1993) Muscling in on gene therapy. Nature News & Views 364:673-675.

59. Rastinejad, F., Conboy, M., Rando, T.A. and Blau, H.M. (1993) Tumor suppression by RNA from the 3' untranslated region of α-tropomyosin. Cell 75:1107-1117.

60. Rando, T.A. and Blau, H.M. (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J. Cell Biol. 125:1275-1287.

61. Mohler, W.A. and Blau, H.M. (1994) Membrane-bound neomycin phosphotransferase confers drug-resistance in mammalian cells: a marker for high-efficiency targeting of genes encoding secreted and cell-surface proteins. Som. Cell Mol. Genet. 3:153-162.

62. Cho, M., Hughes, S.M., Karsch-Mizrachi, I., Travis, M., Leinwand, L.A. and Blau, H.M. (1994) Fast myosin heavy chains expressed in secondary mammalian muscle fibers at the time of their inception. J. Cell Sci. 107:2361-2371.

63. Gussoni, E., Pavlath, G.K., Miller, R.G., Panzara, M.A., Powell, M., Blau, H.M. and Steinman, L. (1994) Specific T-cell receptor gene rearrangments at the site of muscle degeneration in Duchenne muscular dystrophy. J. Immunol. 153:4798-4805.

64. Pavlath, G.K., Rando, T.A. and Blau, H.M. (1994) Transient immunosuppressive treatment leads to long-term retention of allogeneic myoblasts in hybrid myofibers. J. Cell Biol. 127:1923-1932.

65. Rando, T.A., Pavlath, G.K. and Blau, H.M. (1995) The fate of myoblasts following transplantation into mature muscle. Exp. Cell Res. 220:383-389.

66. Dhawan, J., Rando, T.A. Elson, S.L., Bujard, H. and Blau, H.M. (1995) Tetracycline-regulated gene expression following direct gene transfer into mouse skeletal muscle. Som. Cell Mol. Genet. 21:233-240.

67. Blau, H.M. and Springer, M.L. (1995) Overview of gene therapy: a novel form of drug delivery. N. Engl. J. Med. 333:1204-1207.

68. Blau, H.M. and Springer, M.L. (1995) Muscle-mediated gene therapy. N. Engl. J. Med. 333:1554-1556.

69. Hofmann, A., Nolan, G.P. and Blau, H.M. (1996) Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. Proc. Natl. Acad. Sci., USA 93:5185-5190.

70. Gussoni, E., Wang, Y., Fraefel, C., Miller, R.G., Blau, H.M., Geller, A. and Kunkel, L. (1996) A method to codetect introduced genes and their products in gene therapy protocols. Nature Biotechnology 14: 1012-1016.

71. Mohler, W.A. and Blau, H.M. (1996) Gene expression and cell fusion analyzed by lacZ complementation in mammalian cells. Proc. Natl. Acad. Sci., USA 93:12423-12427.

72. Mohler, W.A. and Blau, H.M. (1996) Spectrophotometric quantitation of tissue culture cell number in any medium. BioTechniques 21:260-266.

73. Rohrer, D. and Blau, H.M. (1996) Defective myogenesis in NFB-s mutant associated with a saturable suppression of myf5 activity. Som. Cell Mol. Genet. 22:349-361.

74. Yang, J.T., Rando, T., Mohler, W.A., Rayburn, H., Blau, H.M. and Hynes, R. (1996) Genetic analysis of $\alpha_4$integrin function in the development of mouse skeletal muscle. J. Cell Biol. 135:829-35.

75. Dhawan, J. Rando, T.A., Elson, S.E., Lee, F., Stanley, E.R. and Blau, H.M. (1996) Myoblast-mediated expression of colony stimulating factor-1 (CSF-1) in the cytokine-deficient *op/op* mouse. Som. Cell Mol. Genet. 22:363-81.

76. Mohler, W.A. and Blau, H.M. (1997) Rapid plasmid minipreps in microtiter format from culture to gel. BioTechniques 22:388-390.

77. Miller, R.G., Sharma, K.R., Pavlath, G.K., Gussoni, E., Mynhier, M., Ye, P., Lanctot, A.M., Greco, C.M., Steinman, L. and Blau, H.M. (1997) Myoblast Implantation in Duchenne Muscular Dystrophy: the San Francisco study. Muscle and Nerve 20:469-478.

78. Blau, H.M. and Khavari, P. (1997) Gene therapy: progress, problems, and prospects. Nature Medicine 3:612-613.

79. Charlton, C.A., Mohler, W.A., Radice, G.L., Hynes, R.O. and Blau, H.M. (1997) Fusion competence of myoblasts rendered homozygous null for N-cadherin in culture. J. Cell Biol. 138:331-336.

80. Gussoni, E., Blau, H.M. and Kunkel, L. (1997) The fate of individual myoblasts following transplantation into muscles of DMD patients. Nature Medicine 3:970-977.

81. Rossi, F., Charlton, C.A. and Blau, H.M. (1997) Monitoring protein-protein interactions in intact eukaryotic cells by β-galactosidase complementation. Proc. Natl. Acad. Sci., USA 94:8405-8410.

82. Springer, M.L. and Blau, H.M. (1997) High-efficiency retroviral infection of primary myoblasts. Som. Cell Mol. Genet. 23:203-209.

83. Kang, S-M., Hofmann, A., Le, D., Springer, M.L., Stock, P.G. and Blau, H.M. (1997) Immune response and myoblasts that express Fas ligand. Science 278:1322-1324.

84. Hofmann, A. and Blau, H.M. (1997) Death of solid tumor cells induced by Fas ligand-expressing primary myoblasts. Som. Cell Mol. Genet. 23:249-257.

85. Rossi, F.M.V. and Blau H.M. (1998) Recent advances in inducible gene expression systems. Current Opinions in Biotechnology 9:451-456.

86. Kringstein, A.M., Rossi, F.M.V., Hofmann, A. and Blau, H.M. (1998) Graded transcriptional response to different concentrations of a single transactivator. Proc. Natl. Acad. Sci., USA 95:13670-13675.

87. Rossi, F.M.V., Guicherit, O.M., Spicher, A., Kringstein, A.M., Fatyol, K., Blakely, B.T. and Blau, H.M. (1998) Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. Nature Genetics 20:389-393.

88. Fridman, J. S., Rehemtulla, A., Hofmann, A., Blau, H.M. and Maybaum, J. (1998) Expression of Bcl-XS alters cytokinetics and decreases clonogenic survival in K12 rat colon carcinoma cells. Oncogene 17:2981-2991.

89. Spicher, A., Guicherit, O.M., Duret, L., Aslanian, A., Sanjines, E.M., Denko, N.C., Giaccia, A.J. and Blau, H., (1998) Highly conserved RNA sequences that are sensors of environmental stress. Mol. Cell. Biol. 18:7371-7382.

90. Springer, M.L., Chen, A. S., Kraft, P. E., Bednarski, M. and Blau, H. M. (1998) VEGF gene delivery to muscle: Potential role for vasculogenesis in adults. Molecular Cell 2:549-558.

91. Blau, H.B., and Rossi, F.M. (1999) Tet B or not tet B: Advances in tetracycline-inducible gene expression. Proc. Natl. Acad. Sci., USA 96:797-799.

92. Springer, M.L., Kraft, P.E. and Blau, H.M. (1999) Inhibition of solid tumor growth by Fas ligand-expressing myoblasts. Som. Cell Mol. Genet. 24:281-289.

93. Estellés, A., Charlton, C.A. and Blau, H.M. (1999) The phosphoprotein protein PEA15 inhibits Fas- but increases TNFR-1-mediated caspase-8 activity and apoptosis. Dev. Biol. 216:16-28.

94. Abendroth, A., Slobedman, B., Springer, M.L, Blau, H.M. and Arvin, A.M. (1999) Analysis of immune responses to varicella zoster viral proteins induced by DNA vaccination. Antiviral Research 44:179-192.

95. Springer, M.L., Ip, T.K. and Blau, H.M. (2000) Angiogenesis monitored by perfusion with a space-filling microbead suspension. Mol. Therapy 1:82-87.

96. Rossi, F.M.V., Blakely, B.T. and Blau, H.M. (2000) Interaction blues: Protein interactions monitored in live mammalian cells by β–gal complementation. Trends in Cell Biology 10:119-122.

97. Blakely, B.T., Rossi, F. M.V., Tillotson, B., Palmer, M., Estellés, A. and Blau, H.M. (2000) Epidermal growth factor receptor dimerization monitored in live cells. Nature Biotech. 18:218-222.

98. Charlton, C.A., Mohler, W.A. and Blau, H.M. (2000) Neural cell adhesion molecule (NCAM) and myoblast fusion. Devel. Biol. 221:112-119.

99. Springer, M.L., Hortelano, G., Bouley, D., Wong, J., Kraft, P.E. and Blau, H.M. (2000) Induction of angiogenesis by implantation of encapsulated primary myoblasts expressing vascular endothelial growth factor. J. Gene Med. 2:279-288.

100. Lee, R.J., Springer, M.L., Blanco-Bose, W.E., Shaw, R., Ursell, P.C. and Blau, H.M. (2000) VEGF gene delivery to myocardium: Deleterious effects of unregulated expression. Circulation 102:898-901.

101. Rossi, F.M.V., Kringstein, A.M, Spicher, A., Guicherit, O.M. and Blau, H.M. (2000) Transcriptional control: Rheostat converted to on/off switch. Molecular Cell 6:723-728.

102. Brazelton, T.R., Rossi, F. M.V., Keshet, G.I. and Blau, H.M. (2000) From marrow to brain: Expression of neuronal phenotypes in adult mice. Science 290:1775-1779.

103. Blau, H.M. and Banfi, A. (2001) The well-tempered vessel. Nature Medicine 7:532-534.

104. Blanco-Bose, W.E., Yao, C.-C., Kramer, R.H. and Blau, H.M. (2001) Purification of mouse primary myoblasts based on α7 integrin expression. Exp. Cell Res. 265:212-220.

105. Blanco-Bose, W.E. and Blau, H.M. (2001) Laminin-induced change in conformation of pre-existing α7β1 integrin signals secondary myofiber formation. Devel. Biol. 233:148-160.

106. Springer, M.L., Brazelton, T.R. and Blau, H.M. (2001) Not the usual suspects: the unexpected sources of tissue regeneration. J. Clinical Invest. 107:1355-1356.

107. Blau, H.M., Brazelton, T.R. and Weimann, J.M. (2001) The evolving concept of a stem cell: Entity or function? Cell 105:829-841. (invited)

108. Wehrman, T.S., Kleaveland, B.M., Her, J-H., Balint, R. and Blau, H.M. (2002) Protein-protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments. Proc. Natl. Acad. Sci. USA 99:3469-3474.

109. Springer, M.L., Ozawa, C.R. and Blau, H.M. (2002) Transient production of α-smooth muscle actin by skeletal myoblasts during differentiation in culture and following intramuscular implantation. Cell Motility and the Cytoskeleton 51:177-186.

110. Koransky, M.L., Robbins, R.C. and Blau, H.M. (2002) VEGF gene delivery for treatment of ischemic cardiovascular disease. Trends In Cardiovasc. Med. 12:108-114.

111. Munz, B., Hildt, E., Springer, M.L. and Blau, H.M. (2002) RIP2, a checkpoint in myogenic differentiation. Mol. Cell. Biol. 22:5879-5886.

112. Blau, H.M. (2002) A twist of fate. Nature 419:437. (invited)

113. Blau, H.M., Brazelton, T., Keshet, G. and Rossi, F. (2002) Something in the eye of the beholder. Science 298:361-362. (invited)

114. LaBarge, M.A. and Blau, H.M. (2002) Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell 111:589-601.

115. Weimann, J.M., Charlton, C.A., Brazelton, T.R., Hackman, R.C. and Blau, H.M. (2003) Contribution of transplanted bone marrow cells to purkinje neurons in human adult brains. Proc. Natl. Acad. Sci. 100:2088-2093.

116. Springer, M.L., Ozawa, C.R., Banfi, A., Kraft, P.E., Ip, T.K., Brazelton, T.R. and Blau, H.M. (2003) Localized arteriole formation directly adjacent to the site of VEGF-induced angiogenesis in muscle. Mol. Therapy 7:441-9.

117. Brazelton, T.R., Nystrom, M. and Blau, H.M. (2003) Significant differences among skeletal muscles in the incorporation of bone marrow derived cells. Devel. Biol. 262:64-74.

118. von Degenfeld, G., Banfi, A., Springer, M.L. and Blau, H.M. (2003) Myoblast-mediated gene transfer for therapeutic angiogenesis and arteriogenesis. Brit. J. Pharm. 140:620-626.

119. Weimann, J.M., Johansson, C.B., Trejo, A. and Blau, H.M. (2003) Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons in adult brain after BMT. Nature Cell Biol. 5(11):959-966.

120. Corbel, S.Y., Lee, A., Yi, L., Duenas, J., Brazelton, T.R., Blau, H.M. and Rossi, F.M.V. (2003) Contribution of hematopoietic stem cells to skeletal muscle. Nature Medicine 9(12):1528-1532.

121. Jacobi, J., Tam, B.Y.Y., Sundram, U., von Degenfeld, G., Blau, H.M. Kuo, C. J. and Cooke, J.P. (2004) Discordant effects of a soluble VEGF receptor on wound healing and angiogenesis. Gene Therapy 11:302-309.

122. Sen, G., Wehrman, T.S., Myers, J. and Blau, H.M. (2004) Restriction enzyme-generated siRNA (REGS) vectors and libraries. Nature Genetics 36(2):183-189.

123. Ozawa, C.R., Banfi, A., Glazer, N., Thurston, G., Springer, M.L., Kraft, P.E., McDonald, D.M. and Blau, H.M. (2004) Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J. Clin. Invest. 113(4):516-527.

124. Pomerantz, J. and Blau, H.M. (2004) Nuclear reprogramming: A key to regenerative medicine. Nature Cell Biol. 6:810-816.

125. Doyonnas, R., LaBarge, M.A., Sacco, A., Charlton, C. and Blau, H.M. (2004) Hematopoietic contribution to skeletal muscle regeneration by myelomonocytic precursors. Proc. Natl. Acad. Sci. USA 101(37):13507-13512.

126. Palermo, A.T., LaBarge, M.A., Doyonnas, R., Pomerantz, J. and Blau, H.M. (2005) Bone marrow contribution to skeletal muscle: a physiological response to stress. Devel. Biol. 279:336-344.

127. Jacobi, J., Sydow K., von Degenfeld, G., Zhang, Y., Hayan Dayoub, H., Bingyin Wang B., Patterson, A. J., Kimoto, M., Blau, H.M., and Cooke, J.P. (2004) Overexpression of dimethylarginine dimethylaminohydrolase (DDAH) reduces tissue ADMA levels and enhances angiogenesis. Circulation 111(11):1421-1438.

128. Sen, G. and Blau, H.M. (2005) Argonaute 2/RISC resides in cytoplasmic bodies, sites of mammalian mRNA decay. Nature Cell Biol. 7(6):633-636.

129. Banfi, A., von Degenfeld, G. and Blau, H.M. (2005) Critical role of microenvironmental factors in angiogenesis. Curr. Atheroscler. Rep. 7(3):227-234.

130. Wehrman, T.S., Casipit, C.L., Gewertz, N.M. and Blau, H.M. (2005) Enzymatic detection of protein translocation. Nature Methods 2(7):521-527.

131. Sen, G.L., Wehrman, T.S. and Blau, H.M. (2005) mRNA translation is not a prerequisite for small interfering RNA-mediated mRNA cleavage. Differentiation 73:287-293.

132. Brazelton, T.R. and Blau, H.M. (2005) Optimizing techniques for tracking transplanted stem cells *in vivo*. Stem Cells 23(9):1251-1265.

133. Sacco, A., Doyonnas, R., LaBarge, M.A., Hammer, M.M., Kraft, P. and Blau, H.M. (2005) IGF-I increases bone marrow contribution to adult skeletal muscle and enhances the fusion of myelomonocytic precursors. J. Cell Biol. 171(3):483-492.

134. Wehrman, T.S., von Degenfeld, G., Krutzik, P. O., Nolan, G.P. and Blau, H.M. (2006) Luminescent imaging of β-galactosidase activity in living subjects using sequential reporter-enzyme luminescence. Nature Methods 3(4):295-301.

135. Sen, G.L. and Blau, H.M. (2006) A brief history of RNAi: the silence of the genes. FASEB J. 20(9)U:1293-1299.

136. Kutschka, I., Chen, I.Y., Kofidis, T., Arai, T., von Degenfeld, G., Sheikh, A.Y., Hendry, S.L., Pearl, J., Hoyt, G., Sista, R., Yang, P.C., Blau, H.M., Gambhir, S.S. and Robbins R.C. (2006) Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to the improvement of ischemic rat hearts. Circulation 114(1 Suppl):1167-1173.

137. Kutschka, I., Kofidis, T., Chen, I.Y., von Degenfeld, G., Zwierzchoniewska, M., Hoyt, G., Arai, T., Lebl, D.R., Hendry, S.L., Sheikh, A.Y., Cooke, D.T., Connolly, A., Blau, H.M., Gambhir, S.S. and Robbins R.C. (2006) Adenoviral human BCL-2 transgene expression attenuates early donor cell death after cardiomyoblast transplantation into ischemic rat hearts. Circulation 114 (1 Suppl):1174-1180.

138. von Degenfeld, G., Banfi, A., Springer, M.L., Wagner, R.A., Jacobi, J., Ozawa, C.R., Merchant, M.J., Cooke, J.P. and Blau, H.M. (2006) Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. FASEB J., 20(14):2657-9.

139. Wehrman, T.S., Raab, W.J., Casipit, C.L., Doyonnas, R., Pomerantz, J.H. and Blau, H.M. (2006) A system for quantifying dynamic protein interactions defines a role for Herceptin in modulating ErbB2 interactions. Proc. Natl. Acad. Sci. USA, 103(50):19063-19068.

140. Wehrman, T., He, X., Raab, B., Dukipatti, A., Blau, H. and Garcia, K.C. (2007) Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. Neuron 53:25-38.

141. Springer, M.L., Banfi, A., Ye, J., von Degenfeld, G., Kraft, P.E., Saini, S.A., Kapasi, N.K., and Blau, H.M. (2007) Localization of vascular response to VEGF is not dependent on heparin binding. FASEB J. 21 (Express article doi:10.1096/fj.06-7700com:1-12).

142. Kutschka, I., Chen, I.Y., Kofidis, T., von Degenfeld, G., Sheikh, A.Y., Hendry, S.L., Hoyt, G., Pearl, J., Blau, H.M., Gambhir, S.S. and Robbins, R.C. (2007) *In vivo* optical bioluminescence imaging of collagen-supported cardiac cell graphs. J. Heart and Lung Transplant. 26:3:273-280.

143. Zhang, F., Pomerantz, J.H., Sen, G., Palermo, A.T., and Blau, H.M. (2007) Active tissue-specific DNA demethylation conferred by somatic cell nuclei in stable heterokaryons. Proc. Natl. Acad. Sci. USA 104(11):4395-4400.

144. Wan, D.C., Pomerantz, J.H., Brunet, L.J., Kim, J.-B., Chou, Y.-F., Wu, B.M., Harland, R., Blau, H.M. and Longaker, M.T. (2007) Noggin suppression enhances *in vitro* osteogensis and accelerates *in vivo* bone formation. J. Biol. Chem. 282:26450-26459 (Epub July 2, doi10.1074/jbc.M703282200).

145. Keshet, G.I., Tolwani, R.J., Trejo, A., Kraft, P., Doyonnas, R., Clayberger, C., Weimann, J.M., and Blau, H.M. (2007) Increased host neuronal survival and motor function in BMT Parkinsonian mice: Involvement of immunosupression. J. Comparative Neurology 504:690-701.

146. Blum, G., von Degenfeld, G., Merchant, M.J., Blau, H.M., Bogyo, M. (2007) Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. Nature Chem Biol 3(10):668-77.

147. von Degenfeld, G., Wehrman, T.S., Hammer, M. and Blau, H.M. (2007) A universal technology for monitoring GPCR activation *in vitro* and non-invasively in live animals. FASEB J. 21, (Epub Oct. 17, doi 10.1096/fj.07-9597com).

148. Hammer, M.M., Wehrman, T.S., Blau, H.M. (2007) Novel enzyme complementation-based assays for G protein-coupled receptor signaling, FASEB J. 21, (Epub Oct. 17, doi 10.1096/fj.07-8777com).

149. Streetz, K.L., Doyonnas, R., Grimm, D., Jenkins, D.D., Fuess, S., Perryman, S., Lin, J., Trautwein, C., Shizuru, J., Blau, H., Sylvester, K.G., and Kay, M.A. (2007) Hepatic parenchymal replacement by transplanted allogeneic hepatocytes is facilitated by bone marrow transplantation and mediated by CD4 cells. Hepatology HEP-07-0571.R1 *in press*.

Books

1. Kelly, A. and Blau, H.M., eds. (1992) Molecular and Cellular Biology of Gene Expression in Neuromuscular Development and Disease. In UCLA Symposia on Molecular and Cellular Biology, Raven Press, New York.

2. Blau, H.M. and Watt, F., eds. (1993) Cell differentiation. In Current Opinions in Cell Biology, Vol. 5, Current Biology LTD, London.

3. Lanza, R., Blau, H.M., et al., eds. (2003) Handbook of Adult and Fetal Stem Cells, Academic Press, Elsevier, Burlington, MA.

Committee on Ethics Publications

1. Ruark, J.E., Raffin, T.A., and the Stanford University Medical Center Committee on Ethics including H.M. Blau. (1988) Initiating and withdrawing life support. N. Engl. J. Med. 318:25-30.

2. Thomas, J.A., Hamm, T.E., Jr., Perkins, P.L., Raffin, T.A. and the Stanford University Medical Center Committee on Ethics including H.M. Blau. (1988) Animal research at Stanford University. Principles, policies, and practices. N. Engl. J. Med. 318:1630-1632.

3. Greely, H.T., Hamm, T., Johnson, R., Price, C.R., Weingarten, R., Raffin, T., and the Stanford University Medical Center Committee on Ethics including H.M. Blau. (1989) The ethical use of human fetal tissue in medicine. N. Engl. J. Med. 320:1093-1096.

Invited Chapters

1. Kafatos, F.C., Regier, J., Mazur, G.D., Nadel, M.R., Blau, H.M., Petri, W.H., Wyman, A.R., Gelinas, R.E., Moore, P.B., Paul, M., Efstratiadis, A., Vournakis, J.N., Goldsmith, M.R., Hunsley, J.R., Baker, B., Nardi, J., and Koehler, M. (1977) The eggshell of insects: Differentiation-specific proteins and the control of their synthesis and accumulation during development. In Results and Problems in Cell Differentiation (W. Beermann, ed.), Springer-Verlag, Berlin, 8:45-145.

2. Blau, H.M., Webster, C., Chiu, C.-P., Guttman, S., Adornato, B., and Chandler, F.C. (1982) Isolation and characterization of pure populations of normal and dystrophic human muscle cells. In Muscle Development: Molecular and Cellular Control (Pearson, M.L. and Epstein, H.F., eds.) Cold Spring Harbor Laboratory, New York, pp. 543-556.

3. Blau, H.M., Webster, C. and Chiu, C.-P. (1984) Cytoplasmic activation of muscle genes in stable mouse-human heterokaryons. An approach to the study of cell commitment to myogenesis. In Experimental Biology and Medicine (H.M. Eppenberger and J.-C. Perriard, eds.), S. Karger, Basel, 9:34-40.

4. Kedes, L., Blau, H.M., Gunning, P., Ponte, P., Chiu, C.-P., Bains, W., Engel, J. and Guttman, S. (1984) Molecular genetics of human myogenesis. In Experimental Biology and Medicine (H.M. Eppenberger and J.C. Perriard, eds.), S. Karger, Basel, 9:202-210.

5. Blau, H.M., Chiu, C.-P., Pavlath, G.K. and Webster, C. (1985) Muscle gene expression in heterokaryons. In Gene Expression in Muscle (R.C. Strohman and S. Wolf, eds.), Plenum Press, New York, pp. 231-247.

6. Blau, H.M., Webster, C., Pavlath, G.K. and Chiu, C.-P. (1985) Evidence for defective myoblasts in Duchenne muscular dystrophy. In Gene Expression in Muscle (R.C. Strohman and S. Wolf, eds.), Plenum Press, New York, pp. 85-110.

7. Blau, H.M., Pavlath, G.K., Chiu, C.-P., Hardeman, E. and Webster, C. (1986) Activation and expression of muscle genes in heterokaryons. In Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology (C. Emerson, D.A. Fischman, B. Nadal-Ginard, and M.A.Q. Siddiqui, eds.), Alan R. Liss, Inc., New York, 29:105-116.

8. Minty, A.J., Hardeman, E., Gunning, P., Bains, W., Blau, H.M. and Kedes, L. (1986) Regulation of actin gene expression in mammalian striated muscle. In Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology (C. Emerson, D.A. Fischman, B. Nadal-Ginard, and M.A.Q. Siddiqui, eds.), Alan R. Liss, Inc., New York, 29:507-519.

9. Silberstein, L. and Blau, H.M. (1986) Two fetal-specific fast myosin isozymes in human muscle. In Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology (C. Emerson, D.A. Fischman, B. Nadal-Ginard, and M.A.Q. Siddiqui, eds.), Alan R. Liss, Inc., New York, 29:253-262.

10. Webster, C., Filippi, G., Rinaldi, A., Mastropaolo, C., Tondi, M., Siniscalco, M. and Blau, H.M. (1986) Linkage analysis of Duchenne phenotype and G6PD isotype in myoblasts from doubly heterozygous carriers. In Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology (C. Emerson, D.A. Fischman, B. Nadal-Ginard, and M.A.Q. Siddiqui, eds.), Alan R. Liss, Inc., New York, 29:911-919.

11. Blau, H.M., Pavlath, G.K., Hardeman, E.C., Chiu, C.-P., Silberstein, L., Webster, S.G., Miller, S.C., and Webster, C. (1986) Plasticity of the differentiated state. In Biotechnology: The Renewable Frontier (D.E. Koshland, Jr., ed.), The American Association for the Advancement of Science, Washington, D.C., pp. 143-160.

12. Bloch, E. and Blau, H.M. (1979; revised 1987) The family model with genetic counseling. In Marital and Family Therapy (Third Edition) (I.D. Glick, J.F. Clarkin, and D.R. Kessler, eds.), Grune and Stratton, Inc., Orlando, Florida.

13. Ham, R.G., St. Clair, J.A., Blau, H.M. and Webster, C. (1989) Serum-free media for growth and differentiation of human muscle satellite cells. In Cellular and Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology, new series, vol. 93 (L. Kedes and F. Stockdale, eds.), Alan R. Liss, Inc., New York, pp. 905-914.

14. Blau, H.M., Pavlath, G.K., Chiu, C.-P., Hardeman, E., Miller, S., Blakely, B., Pan, L., Peterson, C. and Schäfer, B.W. (1989) Heterokaryons: A model system for studying cell commitment and differentiation. In Cellular and Molecular Biology of Muscle Development, UCLA Symposia on Molecular and Cellular Biology, new series, vol. 93 (L. Kedes and F. Stockdale, eds.), Alan R. Liss, Inc., New York, pp. 851-861.

15. Hughes, S.M. and Blau, H.M. (1990) Regulation of regional specialization in muscle fibers. In The Dynamic State of Muscle Fibers (D. Pette, ed.), Walter de Gruyter and Co., Berlin, pp. 265-277.

16. Blau, H.M., Webster, C. and Pavlath, G.K. (1990) Purification and proliferation of human myoblasts isolated with fluorescence activated cell sorting. In International Conference on Myoblast Transfer Therapy (R. Griggs and G. Karpati, eds.), Plenum Press, New York, pp. 97-100.

17. Blau, H.M., Pavlath, G.K., Rich, K. and Webster, S.G. (1990) Localization of muscle gene products in nuclear domains: Does this constitute a problem for myoblast therapy? In International Conference on Myoblast Transfer Therapy (R. Griggs and G. Karpati, eds.), Plenum Press, New York, pp. 167-172.

18. Blau, H.M. and Hughes, S.M. (1990) Retroviral lineage markers for assessing myoblast fate *in vivo*. In International Conference on Myoblast Transfer Therapy (R. Griggs and G. Karpati, eds.), Plenum Press, New York, pp. 201-203.

19. Blau, H.M. and Hughes, S.M. (1990) Cell lineage in vertebrate development. In Current Opinion in Cell Biology 2:981-985.

20. Blau, H.M., Webster, C., Pavlath, G.K. (1990) Purification and Proliferation of Human Myoblasts Isolated with Flourecence Activated Cell Sorting Advances in Experimental;

Medicine and Biology, vol. 280 (R. Griggs, G. Karpati, eds) Plenun Publishing Corp., New York, NY, pp. 97-100.

21. Miller, R.G., Mhaundar, S., Steinman, L., Almada, A., Pavlath, G.K., and Blau, H.M. (1991) Methodologic considerations in clinical studies of myoblast implantation. In Excerpta Medica International Congress Series (C. Angelini, G.A. Danieli, D. Fontanari eds.), Elsevier Science, Amsterdam, Holland, pp. 117-122.

22. Hughes, S.M. and Blau, H.M. (1991) Retroviral vectors as probes of muscle development. In Restorative Neurology (A. Wernig, ed.), Elsevier Science Publishers, pp. 11-14.

23. Blau, H.M., Pavlath, G.K., Gussoni, E., Steinman, L., Miller, R.G., Sharma, K. (1992) Myoblast transfer in DMD: Problems in the interpretation of efficiency: A reply. Letter to the Editor, Muscle and Nerve 15:1209-1210.

24. Blau, H.M., Blakely, B.T., Pan, L.C., Schaefer, B.W., Peterson, C.A., Rastinejad, F., Rohrer, D., and Hwung, Y.-P. (1992) A genetic approach to cloning regulators of mammalian differentiation. In Molecular and Cellular Biology of Gene Expression in Neuromuscular Development and Disease, UCLA Symposia on Molecular and Cellular Biology (A. Kelly, H.M. Blau, eds.), Raven Press, New York, pp.115-129

25. Blau, H.M. (1992) Regulating the myogenic regulators. In The Molecular Biology of Muscle, Society for Experimental Biology, Symposium No. 46 (A. El Haj, ed.), Cambridge University Press, pp. 9-18.

26. Blau, H.M. (1993) Plasticity of the differentiated state. In Gene Expression: General and Cell-Type-Specific (M. Karin, ed.), Birkhauser, Boston, pp. 25-42.

27. Blau, H.M., Pavlath, G.K. and Dhawan, J. (1993) Myoblast mediated gene therapy. In Tissue Engineering (E. Bell, ed.), Birkhauser, Boston, pp. 37-47.

28. Blau, H.M. (1994) Heterokaryons reveal that differentiation requires continuous regulation. In The Legacy of Cell Fusion (S. Gordon, ed.), Oxford University Press, Oxford, pp. 3-16.

29. Dhawan, J. and Blau, H.M. (1995) Systemic delivery of recombinant proteins by genetically engineered myoblasts. In Trends and Future Perspectives in Peptide and Protein Drug Delivery (V.H.L. Lee, M. Hashida, Y. Mizushima, eds.), Harwood Academic Publishers GMBH, Switzerland, pp. 351-364.

30. Springer, M.L., Rando, T.A. and Blau, H.M. (1997) Gene delivery to muscle. In Current Protocols in Human Genetics, Unit 13.4 (A.L. Boyle, ed.), John Wiley & Sons, Inc., New York, pp. 13.4.1-13.4.19. [2nd edition: 2001), (A.L. Boyle and A. Rosenzweig, eds.), pp. 13.4.1-13.4.21].

31. Rando, T.A. and Blau, H.M. (1997) Methods for myoblast transplantation. In Methods in Muscle Biology, Methods in Cell Biology series, vol. 52 (C.P. Emerson, H.L. Sweeney, eds.), Academic Press, San Diego, pp. 261-272.

32. Blau, H.M. and Blakely, B.T. (1999) Plasticity of cell fate: Insights from heterokaryons. In Seminars in Cell & Developmental Biology, vol. 10. (A. Colman, ed.) Academic Press, San Diego, pp. 267-272.

33. Ozawa, C.R., Springer, M.L. and Blau, H.M. (2000) *Ex vivo* gene therapy using myoblasts and regulatable retroviral vectors. In Gene Therapy: Therapeutic Mechanisms and Strategies. (N.S. Templeton, D.D. Lasic, eds.) Marcel Dekker, Inc., New York, pp. 61-80; 2nd edition (2004) In Gene and Cell Therapy: Therapeutic Mechanisms and Strategies (N.S. Templeton, ed.), pp. 147-164.

34. Rossi, F.M.V., Blakely, B.T., Charlton, C.A. and Blau, H.M. (2000) Monitoring protein-protein interactions in live mammalian cells by β-galactosidase complementation. In Applications of Chimeric Genes and Proteins. Methods in Enzymology, vol. 328. (J.N. Abelson, S.D. Emr, J. Thorner, eds.) Academic Press, San Diego, pp. 231-251.

35. Ozawa, C.R., Springer, M.L. and Blau, H.M. (2000). A novel means of drug delivery: myoblast-mediated gene therapy and regulatable retroviral vectors. In Annual Review of Pharmacology and Toxicology 40. (A.K. Cho, ed.) Annual Reviews, Inc., Palo Alto, pp. 295-317.

36. Blakely, B.T., Rossi, F.M.V., Wehrman, T.S., Charlton, C.A. and Blau, H.M. (2002) Protein interactions in live cells monitored by β-galactosidase complementation. In Protein-Protein Interactions: A Molecular Cloning Manual, (E. Golemis, ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 407-414.

37. Blau, H.M. and Rossi, F.M.V. (2002) Regulatable expression. In Encyclopedia of Molecular Medicine, vol 5. (T.E. Creighton, ed.) John Wiley & Sons, Inc., New York, pp. 2747-2750.

38. Banfi, A, Springer M.L. and Blau, H.M. (2002) Myoblast-mediated gene transfer for therapeutic angiogenesis. In Gene Therapy Methods. Methods in Enzymology (M.I., Phillips, ed.) Academic Press, San Diego, pp. 145-157.

39. Doyonnas, R. and Blau, H.M. (2003) What is the future of stem cell research? Whether entity or function? In Stem Cells Handbook, (S. Sell, ed.) Humana Press Inc., Totowa, NJ, pp. 491-499.

40. Brazelton, T.R. and Blau, H.M. (2004) Plasticity of circulating adult stem cells. In Lung Development and Regeneration, (D. Massaro, ed.) Marcel Dekker, Inc., New York, NY, pp. 217-246.

41. LaBarge, M.A. and Blau, H.M. (2005) Skeletal muscle stem cells. In Handbook of Stem Cells, Vol. 2: Adult and Fetal Stem Cells, (R. Lanza, H. Blau, et al., eds.) Academic Press, Elsevier, Burlington, MA, pp. 211-216.

42. Blau, H.M. (2005) Adult stem cells: prospects for reprogramming bone marrow derivatives in stable heterokaryons. In Commemorative volume of the 400th Anniversary of the Pontifical Academy of Sciences, *in press*.

43. Pomerantz, J.H. and Blau, H.M. (2007) Skeletal muscle stem cells. In Principles of Regenerative Medicine, (A. Atala, R. Lanza, J.A. Thomson, R.M. Nerem, eds.) Elsevier Academic Press, pp.376-387, *in press*.

Invited Presentations (1990-present):

1990 Special Lecture in Molecular and Cellular Approaches to Cardiovascular Diseases, Program of Excellence in Molecular Biology, University of California, San Francisco, CA
Plenary Speaker, Inaugural Symposium MGH Cancer Center on Cell Cell Interactions and Cell Fate Decisions, Boston, MA
Seminar, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA
Seminar, Department of Biology, Princeton University, Princeton, NJ
Plenary Speaker, The Fifth ICN-UCI Symposium on Molecular Aspects of Development, University of California, Irvine, CA
Plenary Speaker, UCLA/Keystone Symposium on Tissue Engineering Session Chair, Muscle Replacement and Maintenance, Keystone, CO
Special Lecture, 15th International Cancer Congress, Hamburg, Germany
Co-Chair with Michael Karin, FASEB Meeting, Regulation of Tissue Specific Gene Expression, Copper Mountain, CO
Plenary Speaker, Symposium on Molecular Genetics and Future Concepts of Therapy, VII International Congress on Neuromuscular Diseases, Munich, Germany
Jacques Monod Conference, Cell Interactions and Cell Differentiation, Toulon, France
Chair and Speaker, Minisymposium, American Society for Cell Biology, Control of Developmental Decisions in Muscle, San Diego, CA

1991 Co-organizer with Alan Kelly, and Plenary Speaker, ICN-UCLA Symposium: Gene Expression in Neuromuscular Development, Keystone, CO
Plenary Speaker, Graduate Student Symposium, Department of Molecular, Cellular and Developmental Biology, Boulder, CO
Distinguished Lecture Series on Molecular Biology in Medicine, Rutgers University, Piscataway, NJ
Bing Luncheon, Stanford Medical Development Lecture Series, Los Angeles, CA
Gene Therapy Program, Howard Hughes Medical Institute, Departments of Internal Medicine and Human Genetics, University of Michigan Medical Center, Ann Arbor, MI
Seminar, Department of Developmental and Cell Biology, University of California, Irvine, CA
Biochemistry Department Mini-course on the Mouse as an Experimental System: New Methods of Manipulation, University of California, San Francisco, CA
Plenary Speaker, International Symposium: Biotechnology of Growth Factors, Milano, Italy
Seminar, Department of Anatomy and Cell Biology, Harvard Medical School, Boston, MA
Seminar, Department of Biology, Harvard University, Cambridge, MA
Keynote Address, Gordon Conference on Neural Plasticity, Heniker, NH
Plenary Speaker, Symposium of Society of Experimental Biology, Birmingham, England
Gene Therapy Conference, Muscular Dystrophy Association, Tucson, AZ
Stanford Centennial, Stanford University, Stanford, CA

Delegation of U.S. National Academy of Sciences; Plenary Speaker, Joint Symposium on Gene Expression and Gene Regulation, Shanghai, China
Plenary Speaker, International Conference on the Molecular and Cellular Biology of theCardiac Myocyte, American Heart Association, Asilomar, CA
Plenary Speaker, The First IMSUT International Symposium for Biomedical Research: Growth factors, signal transduction, and development, Tokyo, Japan

1992 Plenary Speaker, Keystone Symposium: Transcriptional Regulation in Development, Differentiation and Disease, Tamarron, CO
Seminar, Cell and Molecular Biology, Lawrence Berkeley Laboratory, Berkeley, CA
Plenary Speaker, Keystone Symposium: Growth and Differentiation Factors in Vertebrate Development, Keystone, CO
Pediatric Ground Rounds, Stanford University, Stanford, CA
Medicine Grand Rounds, Stanford University, Stanford, CA
Plenary Speaker, Women in Medicine Conference, Stanford University, Stanford, CA
Plenary Speaker FASEB Meeting: Transcriptional Regulation: Differentiation, Development and Disease, Copper Mountain, CO
Plenary Speaker, 5th International Congress on Cell Biology, Madrid, Spain
Plenary Speaker, EMBO Workshop-Molecular Biology and Pathology of Skeletal and Cardiac Myogenesis, Sardinia, Italy
Plenary Speaker, Gene Therapy, IBC Conference, Washington, D.C.
Plenary Speaker, Henry Harris Symposium: The legacy of cell fusion, Oxford, U.K.
Plenary Speaker, Symposium on Modern Research, Convocation of Queen Lane Building, Medical College of Pennsylvania, PA
Plenary Speaker, Oncogene Science Transcription Symposium, Cold Spring Harbor, NY
Plenary Speaker, Johnson & Johnson Gene Therapy Bio-Science Advisory Committee Meeting, New Brunswick, NJ

1993 Plenary Speaker, Keystone Symposium: Transcription Factors, Regulation and Differentiation, Keystone, CO
Plenary Speaker, Developmental Biology of the Cardiovascular System, Taos, NM
Dean's Distinguished Speaker, University of Colorado Health Sciences Center, Denver, CO
Plenary Speaker, Keystone Symposium: Gene Therapy, Keystone, CO
Gordon Conference on Biological Structure and Gene Expression, Volterra, Italy
Plenary Speaker, 4th International Symposium, Center for Biotechnology, Gene Transfer and Gene Therapy, Huddinge, Sweden
Plenary Speaker, ALZA 25th Anniversary Symp: Biological Delivery Systems, Stanford, CA
Gordon Conference: Developmental Biology, Proctor Academy, Andover, NH
Chair and Plenary Speaker, Symposium on Cell-Mediated Gene Therapy: A Novel Form of Drug Delivery, American Society for Pharmacology and Experimental Therapeutics, Annual Meeting, San Francisco, CA
Plenary Speaker, Conference on Delivery of Protein Drugs, The Next Ten Years, Kyoto, Japan (Represented by postdoctoral fellow, Dr. J. Dhawan)

Plenary Speaker, University of Chicago Cardiovascular Research Institute Symposium on Gene Therapy, Chicago, IL
Plenary Speaker, Gene Diagnosis and Gene Therapy, German Cancer Research Center, Heidelberg, Germany
Plenary Speaker, The Robert Steel Foundation International Symposium, Memorial Sloan-Kettering Cancer Center, New York, NY
Seminar, La Jolla Cancer Research Foundation, La Jolla, CA
3rd Annual University of California, San Diego American Heart Association Bugher Foundation Seminar Series, La Jolla, CA

1994 Seminars in Biology, University of California, San Francisco, CA
Seminar, Gladstone Institute Seminar Series, San Francisco, CA
Plenary Speaker, Keystone Symposium: Molecular Biology of Human Genetic Disease and Gene Therapy, Copper Mountain, CO
Keynote Speaker, West Coast Developmental Biology Conference, Marshall, CA
Seminar, University of Chicago Seminar Series, Dept. of Molecular Genetics & Cell Biology and Biochemistry & Molecular Biology, Chicago, IL
Plenary Speaker, 16th Annual Symposium: La Jolla Cancer Research Foundation, Stem Cells: Biology and Clinical Utility, La Jolla, CA
Session Chair and Plenary Speaker, Keystone Symposium: Molecular Biology of Muscle Development, Snowbird, UT
Plenary Speaker, American Association for Cancer Research 85th Annual Meeting: Transcriptional Control of Cell Proliferation and Differentiation, San Francisco, CA
McClintock Lecture, Cell and Molecular Biology, University of British Columbia, Canada
Plenary Speaker, 52nd Annual Meeting of the Society for Developmental Biology, University of Wisconsin, Madison, WI
Seminar, Neuromuscular Research Centre, St. Vincent's Hospital, Melbourne, Australia
Plenary Speaker, Gene Therapy Symposium, Cold Spring Harbor, NY
Keynote Address, Society for Basic Urologic Research, Stanford, CA
Seminar, Interdepartmental Seminar Series, UCLA School of Medicine, Los Angeles, CA
Seminar, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA
Seminar, Department of Cell Biology, Harvard Medical School, Boston, MA
Seminar, Department of Cell Biology, Univ. of Massachusetts Medical Center, Worcester, MA
Plenary Speaker, Cellular Disease Mechanisms and Therapeutic Strategies, American Society for Cell Biology 34th Annual Meeting, San Francisco, CA

1995 Seminar: Department of Biochemistry and Molecular Biology, MD Anderson Cancer Center, Houston, TX
Plenary Speaker, Gene Therapy: Moving From The Lab To The Clinic, Strategic Research Institute Conference, San Diego, CA
Session Chair and Plenary Speaker, Keystone Symposium: Gene Therapy and Molecular Medicine, Steamboat Springs, CO (Represented by postdoctoral fellow, Dr. M. Springer)

Special Lectures, Marie Curie Amphitheatre, Institut Curie, Paris, France (series of four lectures (1) gene regulation of the MyoD family of bHLH regulators, (2) gene and cell therapy for Duchenne Muscular Dystrophy, (3) myoblast-mediated gene therapy for nonmuscle disorders, (4) the role of riboregulators in development)
Special Lecture, Institut Pasteur, Paris, France
Special Lecture, Rhone Poulenc Rorer, Inc., Paris, France
Special Lecture, College de France, Institut d'Embryologie Cellulaire et Moleculaire, Nogent sur-Marne, Paris, France
Special Lecture, College de France, Laboratoire de Biochimie Cellulaire, II Place Marcelin Berthelot, Paris, France
Plenary Speaker, 1st European Molecular Biology Laboratory Conference (ECBO): Cell Biology of Development, Heidelberg, Germany
Seminar, Differentiation Programm, EMBL, Heidelberg, Germany
Co-organizer with N. Rosenthal and Plenary Speaker, 54th Annual Meeting of the Society for Developmental Biology: Genes, Development, Cancer, San Diego, CA
Visiting Professor and Special Lectureship, Southwestern Medical Foundation For Women in Science and Medicine, University of Texas, Dallas, TX
Plenary Speaker, San Francisco Symposium 95: Translation and Stability of mRNA, San Francisco, CA
19th Annual Mildred Trotter Lecture, Washington University School of Medicine, St. Louis, MO
Plenary Speaker, Pennsylvania Muscle Institute Retreat and Symposium, University of Pennsylvania Medical Center, Philadelphia, PA
Organizer, ALZA Gene Therapy Workshop, Palo Alto, CA
Special Seminar, Wellcome/CRC Institute, Cambridge, England
Medical Nobel Committee, Special Lectures, Karolinska Research Series, Stockholm, Sweden

1996 Session Chair and Plenary Speaker, Keystone Symposium: Molecular Biology of the Cardiovascular System, Keystone, CO
Banbury Conference Meeting: Cellular and Molecular Biology of Mesenchyme, Cold Spring Harbor, NY
Distinguished Lecture Series, Division of Biological Sciences, Harvard School of Public Health, Boston, MA
Seminar, Cancer Center, Massachusetts Institute of Technology, Boston, MA
Seminar, Cell Biology Department, Duke University Medical Center, Durham, NC
Office of Medical Development Special Program: Gene Therapy - Challenge and Promise of a New Frontier, Stanford University, Stanford, CA
Seminar, Genetics and Molecular Medicine Seminar Series, Emory University, Atlanta, GA
Honors Program Lecture, New York University School of Medicine, New York, NY
Plenary Speaker, Stanford's Beckman Center 7th Annual Program in Molecular Genetic Medicine Symposium: Genetics to Gene Therapy for Complex Cardiovascular and Metabolic Disease, Stanford University, Stanford, CA
Plenary Speaker, Conference on Gene Therapy, Genomic Science Series, Hilton Head, SC

Seminar, UCLA School of Medicine, Los Angeles, CA
Plenary Speaker on Gene Therapy, Oncology Conference, Institut Curie, Paris, France
Basement Membrane Gordon Conference, Henniker, NH
Plenary Speaker, Human Gene Therapy Meeting, Cold Spring Harbor, NY (Represented by postdoctoral fellow, Dr. A. Hofmann)
Plenary Speaker, Third International Congress of the Cell Transplant Society, Miami, FL (Represented by postdoctoral fellow, Dr. M. Springer)
Session Co-chair and Plenary Speaker of the "RNA World", Sixth International Congress on Cell Biology, San Francisco, CA

1997 Co-Organizer with James Wilson, Keystone Meeting: Molecular and Cellular Biology of Gene Therapy, Snowbird, UT
Plenary Speaker, Keystone Meeting: Molecular Biology of Muscle Development, Snowmass, CO
Plenary Speaker, Jacques Monod Conference, Aussois, France (Represented by postdoctoral fellow, Dr. A. Spicher)
EMBO Workship on Viral Vectors in Basic Biology, Heidelberg, Germany (Represented by postdoctoral fellow, Dr. O. Guicherit)
Invited Speaker, Bellagio Study Center of Rockefeller Foundation, Lake Como, Italy
Plenary Speaker, Presidential Symposium, American College of Neuropsychopharmacology, Hawaii

1998 Plenary Speaker, Keystone Meeting: Molecular and Cellular Biology of Gene Therapy, Keystone, CO
Plenary Speaker, Symposium for Molecular Medicine: The Road Toward Human Gene Therapy, University of California, San Diego, CA
Plenary Speaker, Barbara H. Bowman Memorial Symposium, The University of Texas Health Science Center at San Antonio, TX
Moderator and Session Chair, First Annual Meeting of the American Society of Gene Therapy, Seattle, WA
Plenary Speaker, 57th Annual Meeting, American Society for Developmental Biology, Stanford, CA
Invited Speaker, Gordon Research Conference on Myogenesis, Tilton, NH
Plenary Speaker, Molecular Interaction Technologies '98, San Francisco, CA
Special Invited Lecture, Amgen, Inc., Thousand Oaks, CA

1999 Plenary Speaker, Keystone Symposium on Molecular and Cellular Biology of Gene Therapy, Salt Lake City, UT
Invited Speaker, Chair Search Committee for Pharmacology Department, Cancer Center, Yale University, New Haven, CT
Invited Speaker, Cardiovascular Medicine Research & Clinical Conferences (CVRC), "Vascular Endothelial Growth Factor (VEGF): Problems and Prospects for Gene Therapy", Stanford University

Invited Speaker, Conference on Stem Cells, Banbury Center, Cold Spring Harbor Laboratory, NY
Medical Scientist Training Program Invited Lecturer, Medical College of Wisconsin, Milwaukee, WI
Invited Speaker, American Society for Pharmacology and Experimental Therapeutics (ASPET) Teaching Institute on Pharmacologists of the Future, Washington, DC
Invited Speaker, American Society for Pharmacology and Experimental Therapeutics (ASPET) Women in Pharmacology Special Lecture (FASEB Excellence in Science Award), Wash., DC
Plenary Speaker, North American Vascular Biology Organization (NAVBO), Tissue Remodeling in Angiogenesis Symposium, Washington, DC
Distinguished Woman in Medicine and Science Lecturer, Northwestern University, Chicago, IL
Keynote Speaker, Student Research Forum, Oregon Health Sciences University, Portland, Oregon
Invited Speaker, Molecular, Cellular and Developmental Biology Department, California Institute of Technology, Pasadena, CA
Invited Speaker, Prescription for Discovery, Pharmaceutical Seminar Series, Perkin Elmer Applied Biosystems, San Francisco, CA
Plenary Speaker, Symposium on Cardiovascular Diseases, American Society of Gene Therapy (ASGT), Washington, DC
Chair, Symposium on Integrating Viral Vectors, American Society of Gene Therapy (ASGT), Washington, DC
Invited Speaker, Gordon Research Conference on Wound Repair, Colby-Sawyer College, New London, NH
Invited Speaker, Institute of Medicine (IOM) Distinguished Lecturer, Ann Arbor, MI
Plenary Speaker, 7th Annual Retreat, Institute for Human Gene Therapy, Atlantic City, NJ
FASEB Excellence in Science Award Lecture, American Society for Cell Biology (ASCB) Annual Meeting, Washington, DC
Plenary Speaker, Gene Therapy: Delivering the Medicines of the 21st Century Conference, Nature Biotechnology Conference, Washington, DC
Invited Speaker, Division of Cell and Molecular Medicine Seminar, UCSD, CA

2000 Plenary Speaker, New Opportunities in Aging Research in 2000 and Beyond, Gerontology Society of America, San Francisco, CA
Plenary Speaker, 22nd Princeton Conference on Cerebrovascular Disease, Redwood City, CA
Invited Speaker, NIH Director's Wednesday Afternoon Lecture Series for 1999/2000, Washington, DC
Plenary Speaker, 8th European Society of Gene Therapy (ESGT), Stockholm, Sweden
Plenary Speaker, Keystone Symposia: Gene Therapy: The Next Millenium
Plenary Speaker, Keystone Symposia: Molecular Biology of the Cardiovascular System
Plenary Speaker, 22nd Princeton Conference on Cerebrovascular Disease, Redwood City, CA
Biosciences Distinguished Lecturer, Lawrence Berkeley National Laboratory, Berkeley, CA
Plenary Speaker, 5th Ben May Inst. for Cancer Research Symposium, Univ. Chicago

Plenary Speaker, Molecular Biology of Muscle Development and Disease, Asilomar, CA
Keynote Speaker, 25th Annual Meeting, Society of Cardiovascular and Interventional Radiology, San Diego, CA
Plenary Speaker, 3rd Annual Meeting, American Society of Gene Therapy, Denver, CO
Keynote Speaker, BAM'2000, The Sixth Abano Terme Meeting on Rehabilitation, Padova, Italy
Invited Speaker, Department of Cell Biology, Biological Chemistry and Molecular Pharmacology and Institute of Chemistry and Cell Biology, Harvard University School of Medicine (2000)

2001 Plenary Session Chair and Speaker, Gene Therapy 2001: A Gene Odyssey, Keystone Symposium, Utah
Co-Chair and Speaker, Stem Cell Minisymposium, 41st Meeting of the American Society for Cell; Biology (ASCB), Washington, DC
Broadhurst Distinguished Lecturer, Harvard University, Cambridge, MA
Keynote Speaker, SBF 488 Symposium: Neural and Non-Neural Stem Cells, Heidelberg, Germany
Plenary Speaker, Gordon Research Conference on Angiogenesis and Microcirculation, Salve Regina University, Newport, RI
Plenary Speaker, Myogenesis, Gordon Research Conference, Il Ciocco, Italy

2002 Mayer Lecture in the Life Sciences, Massachusetts Institute of Technology, Cambridge, MA
Mary Elizabeth Garrett Lecturer, Johns Hopkins University School of Medicine, Baltimore, MD
Catherine N. Stratton Lecturer on Critical Issues, Whitehead Institute, Massachusetts Institute of Technology, MA
President's (Harold Varmus) Research Seminar Series Lecturer, Memorial Sloan Kettering Cancer Center, NY

2003 Plenary Speaker, British Society for Developmental Biology, Spring Meeting, University of Warwick, England
Plenary Speaker, Perlmutter Family Symposium on Neurodegenerative Disorders, Harvard University School of Medicine
2003 Annual Scientist in Medicine Lecture, University of Washington
Rolf-Sammet-Fonds Visiting Professorship, University of Frankfurt
Honorary Doctorate, University of Nijmegen, Holland
Speaker, Academie des Sciences de la France at Pontifical Academy, the Vatican in Rome, "Stem Cells and Cloning"

2004 Plenary Speaker, XIIIth International Vascular Biology Meeting, Toronto, Canada
Plenary Speaker, 2nd Annual International Society for Stem Cell Research Meeting, Boston, MA
Discussion Leader and Plenary Speaker, Myogenesis, Gordon Research Conference, Il Ciocco, Italy
President's Lecture, 13th International Society of Differentiation Conference, Honolulu, HI

Keynote Speaker, Allergan Foundation Lectures in Modern Biology and the Howard A. Schneiderman Memorial Bioethics Lecture Series, University of California, Irvine

2005 Louis A. Bloomfield Lecturer, Case Western Research University School of Medicine, Cleveland, OH
Speaker, Inaugural Gurdon Institute Symposium, Wellcome Trust / Cancer Research UK Gurdon Institute, Cambridge, UK
Plenary Speaker and Session Chair, Summer Research Conference: Skeletal Muscle Satellite and Stem Cells, FASEB, Tucson, AZ
Speaker, Senior Ellison Grantee, Colloquium on the Biology of Aging, Ellison Medical Foundation, Woods Hole, MA
Plenary Speaker and Symposium Chair, Heinz Herrmann Symposium: Reprogramming Cell Fate, American Society for Cell Biology, San Francisco, CA
Speaker, Symposium: Stem Cells and Aging – Celebrating the Promise, American Federation for Aging Research, San Francisco, CA
Speaker, Oregon Stem Cell Center Seminar Series, Oregon Stem Cell Center, Portland, OR
Speaker, Fourth Annual Cancer Symposium: Stem Cells and Cancer, Rockefeller University, New York, NY
Speaker, Symposium: Molecular Therapy in the 21st Century, School of Pharmacy at the University of Wisconsin-Madison, Madison, WI
Speaker, Department of Pharmacology, University of California – San Diego, San Diego, CA

2006 Stem Cells and Aging Meeting, National Institute on Aging, Potomac, MD
Frontiers in Myogenesis, Society for Muscle Biology, Pine Mountain, GA
Stem Cells and Cancer, German Cancer Research Center, Heidelberg, Germany
Plenary Session Chair and Speaker, Embryonic and Somatic Stem Cells, German Research Foundation and the German Academy of Sciences Leopoldina, Dresden, Germany
Opening Lecturer, EMBO Conference on the Molecular and Cellular Basis of Regeneration and Tissue Repair, Ascona, Switzerland
Keynote Speaker on Stem Cells for Brain in annual meeting: The Role of Stress in Psychiatric and Medical Disorders, The American College of Psychiatrists, San Juan, Puerto Rico
Friday Evening Lecturer, Colloquium on the Biology of Aging, Ellison Medical Foundation, Woods Hole, MA
Invited Lecturer, Regenerative Cell Therapy, International Symposium of the French Academy of Sciences, Paris, France

2007 Plenary Session Chair and Speaker, Stem Cell Interactions with their Microenvironmental Niche, Keystone Symposium, Colorado
Plenary Speaker, Gordon Research Conference on Myogenesis Conference, Il Ciocco, Italy
Invited Speaker, Swiss Institute for Experimental Cancer Research in Lausanne, Switzerland
Invited Speaker, Friedrich Miescher Institut Student Seminar in Basel, Switzerland